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A NEW MODE OF ENZYMATIC PHOSPHATE TRANSFER*

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(Received for publication, August 4, 1947)

Organic phosphate compounds are involved in so many biological processes that any enzymatic reactions which promote phosphate transfer are of direct interest. It is generally regarded, as pointed out by Green and Colowick (1) in their review of the chemistry and metabolism of the compounds of phosphorus, that when phosphate transfer takes place intermolecularly the participation of adenine nucleotides is essential. The present paper describes an intermolecular phosphate transfer in which adenine nucleotides are apparently not required.

EXPERIMENTAL

Phosphatases acting upon susceptible monophosphate esters have been regarded as setting free equivalent amounts of orthophosphate and hydroxy compound. However, it has now been found that, when some phosphatase preparations of widely differing origins act on nitrophenyl phosphate in the presence of certain alcohols, the expected equivalence is not observed, but the orthophosphate is in deficit. This results from the phosphorylation of the added alcohol concurrent with the breakdown of the nitrophenyl phosphate. Phenolphthalein phosphate or phenyl phosphate can qualitatively replace nitrophenyl phosphate in this reaction. Thus in the case of the phosphatase preparations in question, in addition to the simple hydrolysis of a number of "low energy phosphates," a reaction also occurs that may be accurately described as transphosphorylation. Arguments for and against the existence of a transphosphorylating enzyme as separate from the phosphatase will be reviewed later, but the evidence is so far inconclusive.

Method—The substrates were prepared and the analyses performed, unless otherwise noted, as described in a previous paper on citrus phosphatase (2).

Phosphate Acceptors—In Table I may be found some roughly comparable values of the efficacies of a variety of hydroxy compounds found suitable as phosphate acceptors. The following compounds gave negative results: *t*-butyl alcohol, ethanolamine, lactic acid, malic acid, citric acid, serine, pantothenic acid, ethyl acetoacetate, inositol, ribose, glucose, sucrose, α-methylglucoside, ascorbic acid, and chloral. The efficiency of phosphate transfer is expressed in the last column of Table I and elsewhere in the paper in molar percentage and is defined in practical terms as

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* Enzyme Research Laboratory Contribution No. 108.

$$100 \times \frac{(\text{Molar concentration nitrophenol liberated}) - (\text{molar concentration phosphate liberated})}{\text{Molar concentration nitrophenol liberated}}$$

TABLE I

Enzymatic Transfer of Phosphate from p-Nitrophenyl Phosphate to Various Acceptors

Equal units of enzyme were used in the experiments with the unsubstituted monohydroxy compounds; the enzyme concentration was varied in the other experiments.

Acceptor	Concentration of acceptor, quantity per ml. reaction mixture	Concentration of *p*-nitrophenyl phosphate, per ml. reaction mixture	Reaction time	Nitrophenol liberated, per ml. reaction mixture	Phosphate transferred as molar per cent *p*-nitrophenyl phosphate hydrolyzed*
		mg.	*min.*	γ	
n-Propyl alcohol	71 *microliters*	0.60	49.5	128	60
i-Propyl "	71 "	0.60	49.5	111	31
n-Butyl "	71 "	0.60	49.5	111	59
i-Butyl "	71 "	0.60	49.5	98	49
s-Butyl "	71 "	0.60	49.5	103	36
Allyl "	36 "	0.60	48	130	59
Nitroethanol	63 "	0.52	66.5	171	34
Ethylene chlorohydrin	100 "	0.42	34	57	70
Propylene glycol	71 "	0.60	15	124	70
Ethyl lactate	63 "	0.52	66.5	93	16
Glycerol	71 *mg.*	0.60	89	95	28
Erythritol	36 "	0.60	48	94	17
Mannitol	36 "	0.60	48	86	20

Reaction conditions, temperature 37.5°, pH 5.0, usually 0.024 M acetate buffer; acceptors adjusted to pH 5.0 where necessary. Navel orange juice phosphatase similar to Preparation D (2) was used in all cases, except the propylene glycol experiment in which a similar preparation from Valencia orange juice was employed.

* Phosphate transfers of 6 per cent or less are regarded as zero.

If any general conclusions may be drawn from these experiments, they are as follows: primary alcohols are superior to secondary alcohols, and tertiary alcohols are unsuitable. The presence in the alcohol of an amino or carboxyl group, or both, destroys its ability to act as an acceptor. Polyhydroxy alcohols are suitable if spatial conditions are right, if we judge by the success with the straight chain compounds and the failure with inositol. Carbohydrates are unsuited.

An attempt to produce codecarboxylase from pyridoxal by transphosphorylation under conditions similar to those used for propyl alcohol, as shown in Table I, was not successful. Codecarboxylase was tested for by the tyrosine decarboxylase method of Umbreit, Bellamy, and Gunsalus (3). A resting preparation of *Streptococcus faecalis* R grown on a vitamin B_6-deficient medium served as the apoenzyme. Either phosphorylation did not occur or it occurred in the wrong position in the pyridoxal molecule.

A similar attempt was made to phosphorylate thiamine. Although it was hardly to be expected that the pyrophosphate would be formed by the phosphate transfer reaction, the resulting digest was tested for cocarboxylase activity. It did not promote pyruvic acid decarboxylation by alkaline-washed yeast; nor did it affect the rate of carbon dioxide production when thiamine pyrophosphate was present. To investigate the possibility that the monophosphate was formed, a portion of the digest was treated with sufficient alkaline ferricyanide to convert the thiamine and thiamine phosphate, if present, to the corresponding thiochrome derivatives. Repeated extraction was carried out with butyl alcohol to separate the thiochrome from the thiochrome monophosphate. Fluorophotometric examination of the residual aqueous solutions showed that only insignificant amounts of the thiamine monophosphate could have been present.

Effect of pH on Transfer Reaction—Figs. 1 and 2 show the effect of pH variation with *p*-nitrophenyl phosphate as a donor and with *n*-propanol and methanol, respectively, as acceptors. The ratio of moles of phosphate transferred (to the alcohol) to moles of nitrophenol formed (from nitrophenyl phosphate) is obviously a measure of the extent of transphosphorylation as related to total decomposition of the nitrophenyl phosphate. This ratio increased continuously with decreasing pH, but the absolute quantity of phosphate transferred passed through a pH optimum. This optimum pH seemed to be close to that for the liberation of nitrophenol, but differed from the optimum observed for the liberation of inorganic phosphate.

Fig. 3 shows the hydrolysis of monomethyl phosphate by the same enzyme preparation used in the two foregoing experiments (Figs. 1 and 2). In this case also the quantity of phosphate liberated was reduced by the presence of methanol, but the optimum pH of the reaction was not changed.[1]

Effect of Acceptor Concentration—Increasing the concentration of methanol resulted in an increase in both the transfer ratio and the absolute amount of phosphate fixed up to the point where the alcohol began to depress the cleavage of the nitrophenyl phosphate (Fig. 4).

Acceleration of Substrate Cleavage Due to Acceptor—Concurrent with the

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[1] The accumulation of methyl phosphate is a logical consequence because nitrophenyl phosphate in the usual assay concentration is digested at 6.9 times the rate of methyl phosphate at an equivalent concentration. It should be noted that this concentration of methyl phosphate is considerably below the level necessary for the maximum velocity of hydrolysis by phosphatase.

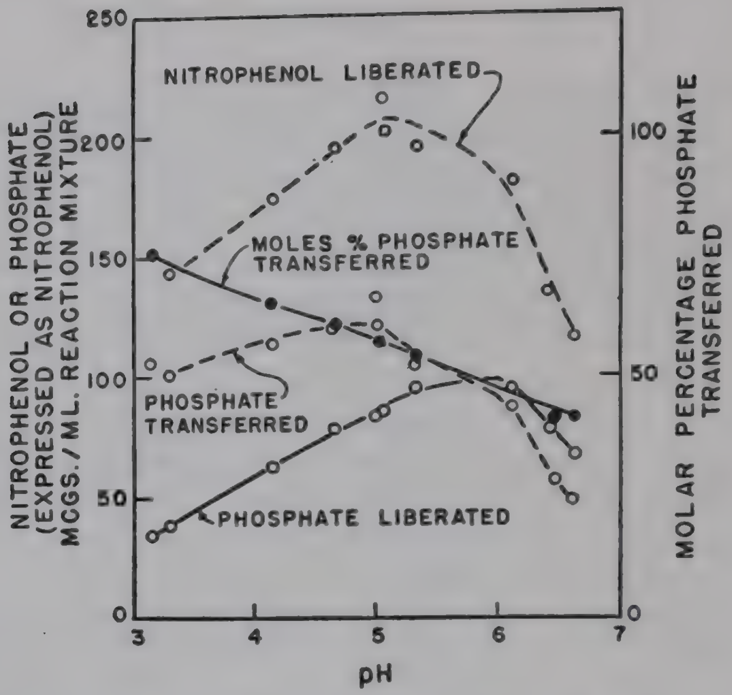

Fig. 1. "Phosphotransferase" action with propanol, as a function of pH. Reaction mixture, 1 ml. of buffer, 5 ml. of water, 5 mg. of disodium nitrophenyl phosphate, 0.5 ml. of *n*-propanol, and 2 ml. of enzyme (dilution of navel orange juice phosphatase Preparation D). Digestion, 74 minutes at 37.5°; acetate-veronal-HCl buffer prepared according to Michaelis (4), except that the sodium chloride solution was omitted.

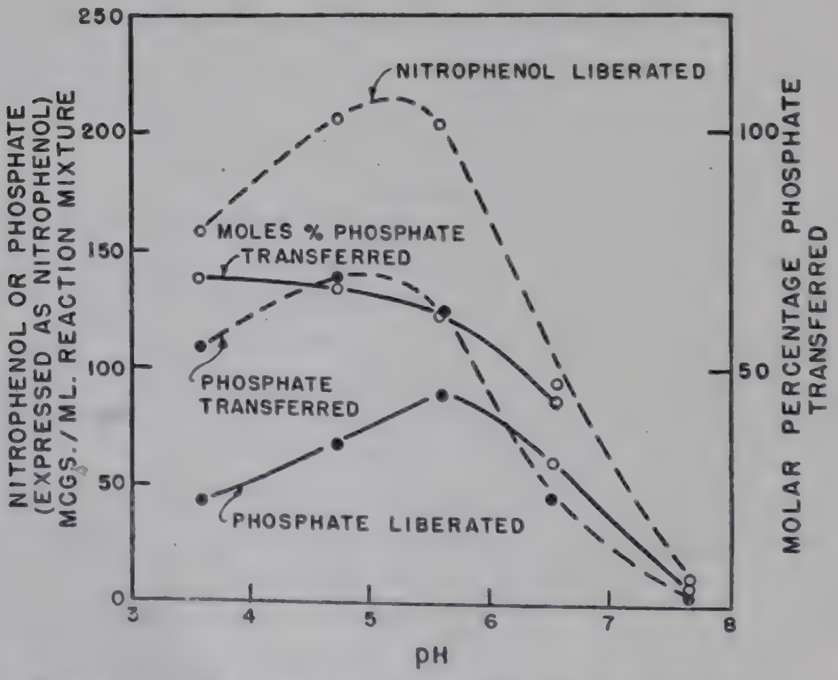

Fig. 2. "Phosphotransferase" action with methanol, as a function of pH. Reaction mixture 1 ml. of buffer, 5 ml. of water, 5 mg. of disodium nitrophenyl phosphate, 1 ml. of methanol, and 1 ml. of the same dilution of enzyme as was used in the experiment shown in Fig. 1. The same buffer system and digestion conditions were also employed.

phosphate transfer occurring when the acceptor was added to the reaction mixture, there was an acceleration of the cleavage of the donor. This effect is seen in Fig. 4, which shows a maximum of 47 per cent increase in hydrolysis caused by the presence of the accepting alcohol. When phenolphthalein phosphate or phenyl phosphate was used as the donor, this acceleration was also noted. The results with phenolphthalein phosphate and ethanol are shown in Table II by way of further illustration. Evidence for the accelerating effect of the alcohol was obtained in virtually all of the experiments in which much transfer occurred, although in some cases of little transfer (for instance with added glycerol) acceleration was not noted. It is obvious that any inhibitory action of the acceptor on the enzymatic cleavage of the donor could mask the stimulatory effect of the transfer reaction.

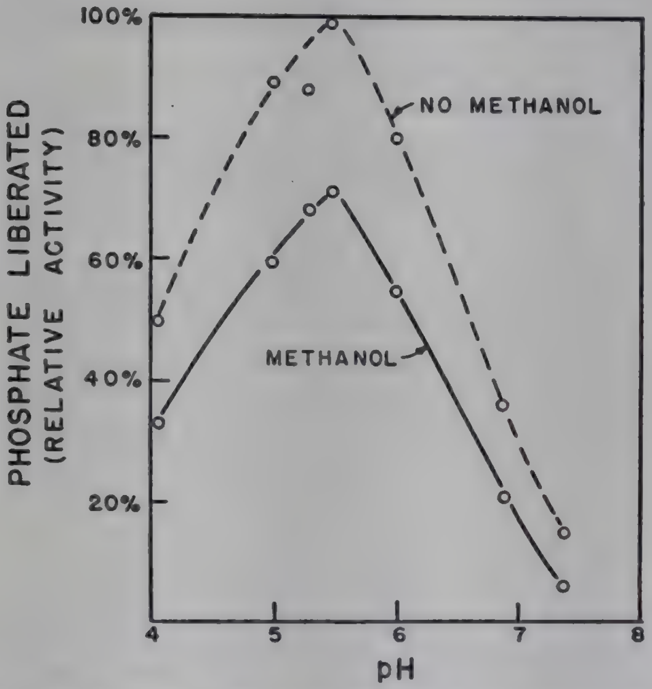

FIG. 3. pH hydrolysis curve of methyl phosphate by the citrus enzyme in the absence and presence of methanol. The activity was measured by determining liberated phosphate. (The relative activity of 100 per cent corresponds to liberation of 42.5 γ of P per tube.) Reaction mixture, 2.5 ml. of 0.0056 M monomethyl phosphate solution, 0.5 ml. of buffer, 0.5 ml. of methanol or water, and 1 ml. of enzyme. The same enzyme and buffer system used in the experiment shown in Fig. 1 were employed here. Digestion, 90 minutes at 37.5°.

Effect of Donor Concentration—Table III shows the variation of phosphate transfer with different concentrations of *p*-nitrophenyl phosphate. Apparently as long as an appreciable concentration of substrate remained the transfer ratio was relatively constant.

Proof of Occurrence of Transfer Reaction—Although the finding that less free phosphate than free nitrophenol was formed in the reaction is good evidence of a transfer of phosphate, it was considered necessary to isolate

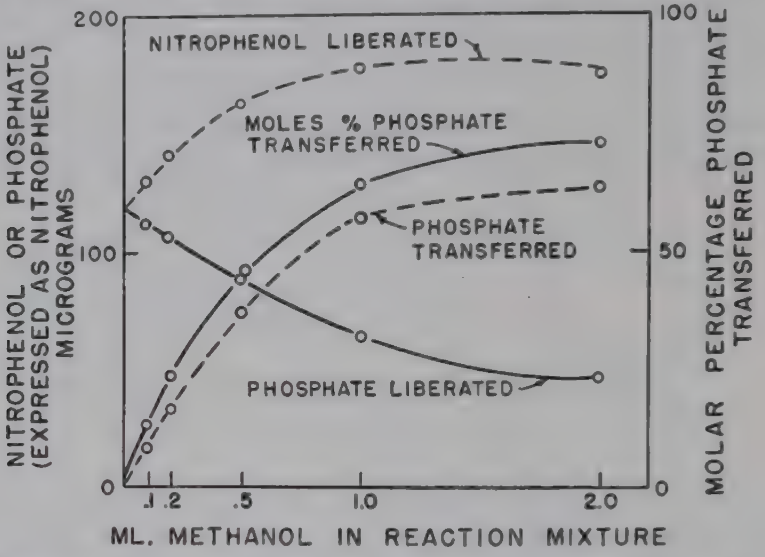

FIG. 4. Effect of acceptor concentration on "phosphotransferase" activity. Substrate, 5 ml. of M/30 acetate, pH 5.15, containing 4.17 mg. of disodium *p*-nitrophenyl phosphate, 2 ml. of enzyme (dilution of navel orange juice phosphatase Preparation D), methanol, and sufficient water to make a final volume of 9 ml. Digestion, 60 minutes at 37.5°.

TABLE II

Effect of Ethanol on Cleavage of Phenolphthalein Phosphate by Citrus Juice Enzyme

Percentage ethanol in reaction mixture	Relative rate of phenolphthalein liberation
0	100
8.50	92
4.25	181
2.13	165
0.83	155

Reaction conditions, temperature 37.5°, digestion time, 50 minutes, assay essentially as described by Huggins and Talalay (5) for acid phosphatase (pH 5.4, 0.001 M phenolphthalein phosphate). Each reaction tube contained 5 ml. of substrate, 0.5 ml. of enzyme (Valencia orange juice phosphatase purified by absorption and elution from diatomaceous earth), ethanol, and sufficient water to make a final volume of 6 ml. In this experiment the enzyme was incubated with the water and ethanol for 1 hour before assaying. Thus the results may include inactivation effects. In all other experiments where an acceptor was used it was introduced prior to the addition of the enzyme.

and identify the acceptor phosphate compound. This was done for monomethyl phosphate by the following procedure.

1 gm. of disodium *p*-nitrophenyl phosphate was dissolved in 25 ml. of

water and the pH adjusted to 5.0 with acetic acid. To this were added 22.2 ml. of methanol, 220 P. N. P. units of navel orange juice phosphatase (Preparation D of an earlier paper (2)), 5 ml. of M acetate buffer, pH 5.0, and water to make 100 ml. After 16 hours incubation at 37.5°, 1390 additional units of enzyme in lyophilized form were added and the incubation continued for 3 hours. At this time 401 mg. of free nitrophenol were present together with 30.6 mg. of inorganic P or 34 per cent of the phosphate equivalent to the nitrophenol. The nitrophenol, together with the residual nitrophenyl phosphate, was removed by six extractions with 150 ml. portions of ether after making the digestion mixture molar with respect to hydrochloric acid. Next, the solution was made pH 8.8 with sodium hydroxide and treated with a calculated excess of barium chloride solution. The precipitate, which contained the inorganic phosphate, was removed by centrifugation and discarded. Ethanol was added to 80 per cent concentration and the precipitate of crude barium methyl phosphate was separated by low speed centrifugation. Pectinaceous material which had accompanied the enzyme settled out quite slowly compared to the barium salt and thus could be removed readily by decantation. The salt was recrystallized several times from water and obtained as thin white crystalline plates which were optically identical with synthetic $Ba(CH_3)PO_4 \cdot H_2O$, prepared by McVicar's method (6).

TABLE III

"Phosphotransferase" Action As Function of Nitrophenyl Phosphate Concentration

p-Nitrophenyl phosphate initially present in reaction mixture	Molar percentage phosphate transfer	Nitrophenol formed in reaction mixture		Extent of digestion of substrate
		Potential	Found	
mg.	*per cent*	*mg.*	*mg.*	*per cent*
50.0	48	23.2	9.3	40
30.0	49.9	13.95	7.94	57
10.0	49.5	4.65	6.06	77
5.0	42.4	2.32	1.93	83
2.5	40.6	1.16	1.19	103

Reaction mixture, 2 ml. of navel orange juice citrus phosphatase (dilution of Preparation D), 0.5 ml. of *n*-propanol, M acetate pH 5.0, the indicated amount of disodium nitrophenyl phosphate · $2H_2O$ and water to make 8 ml. Incubation for 117 minutes at 37.5°.

Reversal of Enzymatic Hydrolysis of Nitrophenyl Phosphate—The reversal of phosphatase hydrolysis, originally reported by Martland and Robison (7), whereby inorganic phosphate is directly esterified to an alcohol, is a well known phenomenon and has been found by many workers to occur with a variety of alkaline phosphatases. Roche (8) has observed a similar behavior in the case of red blood cell phosphatase at pH 6.3. Courtois (9) was unable to find appreciable evidence of synthetic action with several plant phosphatases, except upon employing extremely long incubation periods. Nevertheless, in the case of citrus phosphatase it was necessary to establish that the "phosphotransferase" activity was not actually due to the esterification of the alcohol by the liberated inorganic phosphate. A mixture of 10 ml. of M/30 acetate buffer (pH 5.0), 0.1 ml. of inorganic phosphate (0.0139 mM), 1.0 ml. of methanol, 60 P. N. P. units of Preparation D, and water to make 14 ml. was incubated at 37.5°. No decrease in inorganic phosphate was noted after 3 hours. A repetition of this experiment with the addition of nitrophenol in 2.7×10^{-3} M concentration gave similar results.

"Phosphotransferase" Action in Presence of Added Inorganic Phosphate—If such direct synthetic action had been operative, one might explain the accelerating effect of alcohol as due to the removal of inorganic phosphate which is known to inhibit phosphatase. However, the addition of enough inorganic phosphate to equal that which would have been liberated by complete hydrolysis caused only a 10 per cent decrease in activity, under standard conditions for the assay of nitrophenyl phosphatase.

TABLE IV

Effect of Inorganic Phosphate on Enzymatic Cleavage of Nitrophenyl Phosphate in Presence of Methanol

Inorganic phosphate	Methanol	Relative activity (nitrophenol liberation)
mM	*ml.*	
0.028	0.5	108
0.028	0.0	69
0.0	0.5	149
0.0	0.0	100

The standard assay conditions for "nitrophenyl phosphatase" were employed. All tubes contained 0.014 mM of nitrophenyl phosphate.

The accelerating effect of methanol on the liberation of nitrophenol from nitrophenyl phosphate in the presence and absence of a large excess of inorganic phosphate is shown in Table IV.

"Phosphotransferase" Activity in Other Phosphatase Preparations—Porto Rican Red sweet potato (*Ipomoea batatas*) juice and some fractions obtained therefrom by ammonium sulfate and pH fractionations,[2] although excellent sources of nitrophenyl phosphatase, were entirely devoid of "phosphotransferase" activity; nor did the addition of heat-inactivated citrus phosphatase

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[2] These fractions were obtained in this Laboratory in the course of the isolation of crystalline β-amylase (10).

cause its appearance. A mixture of equal units of sweet potato phosphatase and navel orange juice phosphatase in an alcohol nitrophenyl phosphate substrate behaved essentially as was anticipated on the basis of the independent action of each enzyme (Table V).

TABLE V

Composite Effect of "Phosphotransferase" and "Normal Phosphatase"

Experiment No.	Enzyme	*n*-Propanol	H_2O	*A* Nitrophenol liberated per 0.5 ml. reaction mixture	*B* Phosphate liberated per 0.5 ml. reaction mixture expressed as nitrophenol	Molar percentage phosphate transferred, $\frac{A-B}{A} \times 100$
	ml.	*ml.*	*ml.*	γ	γ	*per cent*
C-Y-1	2, C-Y	0.5	0	97.9	67.1	31.5
C-Y-2	2, "	0	0.5	86.8	88.9	−2
C-1	2, C	0.5	0	104.7	53.6	49.0
C-2	2, "	0	0.5	87.7	85.7	+2
Y-1	2, Y	0.5	0	80.0	80.9	−1
Y-2	2, "	0	0.5	84.9	86.9	−2

	Phosphate or nitrophenol expressed as γ nitrophenol per 5 ml.		
		Calculated	Found
Phosphate liberated in Experiment C-Y-1 due to Enzyme C	26.8		
Phosphate liberated in Experiment C-Y-1 due to Enzyme Y	40.5		
		67.3	67.1
Nitrophenol liberated in Experiment C-Y-1 due to Enzyme C	52.3		
Nitrophenol liberated in Experiment C-Y-1 due to Enzyme Y	40.0		
		92.3	97.9
Molar percentage phosphate transferred in Experiment C-Y-1		27.0 31.5	

The conditions are the same as in the standard nitrophenyl phosphatase assay, except that final volume = 7.5 ml. Reaction time, 72.5 minutes; Enzyme C, dilution of navel orange juice phosphatase Preparation D; Enzyme Y, dilution of sweet potato phosphatase of equal "normal phosphatase" activity; Enzyme C-Y, equal parts of Enzymes C and Y.

Urines of six adults were all capable of effecting the intermolecular transfer of phosphate. Although the normal phosphatase activity of each of the male urines was much higher than that of the female urines, approximately equal transfer ratios were found for both sexes (Table VI).

Alkaline phosphatase, prepared from dog kidney by ethyl acetate-toluene autolysis according to the procedure of Albers and Albers (11), caused no phosphate transfer at pH 9.19 in veronal buffer, regardless of whether Mg^{++} was present or not. Indeed the acceptor, propanol, which was present in a concentration of 7.1 per cent, actually caused a 14 per cent depression in the rate of nitrophenyl phosphate hydrolysis.

Taka-diastase, tested under standard assay conditions in the presence of 7.1 per cent *n*-propanol, gave a transfer ratio of 50 per cent.

Ground tissue preparations were made from onion (Yellow Globe), pear (Bartlett), and apple (Delicious), and similarly tested. Apple caused about a 25 per cent transfer, but onion and pear showed none, although they all possessed normal phosphatase activity.

TABLE VI

"Phosphotransferase" Activity of Human Urine

Sex	"Normal" nitrophenyl phosphatase per ml.	Molar percentage transfer of phosphate
	P. N. P. units	*per cent*
♀	1.7	28
♀	4.6	34
♀	3.0	41
♂	11.8	36
♂	7.0	36.5
♂	16.6	40

"Normal" phosphatase activity was determined by the standard assay method. The transfer was determined at 37.5°. 1 ml. of dialyzed urine was added to a mixture of 5 ml. of M/15 acetate buffer, pH 5.0, containing 4.17 mg. of disodium *p*-nitrophenyl phosphate plus 1 ml. of *n*-propanol.

Non-Participation of Nucleotides—The reasons for dismissing the possibility that nucleotide substances are involved in this reaction are as follows: The citrus phosphatase Preparation D was found practically free of P in the concentrations used. Spectrographic examination in the ultraviolet region showed no absorption characteristic of nucleotides. If the quantity of nucleotide present was so small as to be beyond the limits of measurement, it would have to be used over and over again and would require regeneration by an accompanying oxidation of some substrate not hitherto recognized as suitable for such purposes. However, the transfer reaction was found to occur anaerobically quite as well as in the presence of air. In the latter case, when the transfer was carried out by employing the Warburg technique, no gas exchange was detected. Finally the ability of Preparation D to hydrolyze adenine nucleotide (2) makes it unlikely that this compound could be long maintained in its presence.

DISCUSSION

There can be no doubt that the intermolecular transfer of phosphate can occur without the participation of nucleotide substances but of course it remains to be proved that such a reaction has any biological significance.

Phosphatases are classified on the basis of specificity and pH optimum. The criterion of "phosphotransferase" activity is suggested as a further means of classification. For instance, sweet potato phosphatase and citrus phosphatase, which would probably be regarded as quite similar by ordinary criteria, are distinctly different on the "phosphotransferase" basis.

If the transphosphorylation function described here is not taken into account, some anomalous results can be observed in the study of phosphatase preparations. If nitrophenol phosphatase activity were measured by determination of liberated chromogen, alcohol would appear to accelerate it, whereas, if the liberation of phosphate were taken as an index, the opposite conclusion would be indicated. Further, alcohol would have little influence upon the pH optimum in the former instance, but in the latter the pH optimum would appear to shift toward the alkaline region.

Any attempt to explain completely the mechanism of transphosphorylation requires knowledge of whether the enzyme is identical with phosphatase. Certainly phosphatases exist which cannot perform phosphate transfer, but transfer activity has not yet been found in the absence of normal hydrolytic activity. Partial heat inactivation and fractional fluoride inhibition of citrus juice phosphatase preparations tend to support the theory of a single enzyme. The addition of more phosphatase activity (as sweet potato phosphatase, which does not perform this phosphate transfer) to a preparation high in transfer ability led to results (Table V) which are compatible with the view that a single enzyme is responsible.

Although the question of whether one or more enzymes is involved cannot be considered settled, it seems more likely that the transfer reaction is a property of the moiety that catalyzes the normal hydrolytic reaction. The transfer reaction might then occur in one of two ways; the enzyme-donor complex reacts directly with the alcohol, or the phosphate radical is released in a highly active state and then combines with the alcohol. If the latter conjecture is correct, then it is curious that all acid phosphatases do not act as transfer catalysts. The former hypothesis thus appears more reasonable, and hence the difference between a phosphatase that transfers phosphate and one that does not is probably due to a difference in their specificities toward the solvolytic reagent.

Attention is called to the observation of Rabaté (12) that the β-glucosidase of *Salix purpurea*, which cleaves glucose from β-hydroxyacetophenone glucoside under ordinary conditions, forms β-methyl glucoside when methanol is present during the cleavage. It may be that similar "transferase" phenomena will be found to be of general occurrence with other types of hydrolases.

SUMMARY

1. The intermolecular transfer of phosphate can be performed enzymatically without the participation of nucleotidic compounds.

2. Enzymes capable of this action have been found in citrus fruit, apple, human urine, and taka-diastase, but not in onion, sweet potato, pear, or dog kidney alkaline phosphatase preparation.

3. "Phosphotransferase" has not been obtained free of acid phosphatase and may be an integral property of some acid phosphatases, but certainly not of all.

4. The transfer occurs between aryl phosphates and aliphatic hydroxy compounds. The phosphates of phenol, phenolphthalein, and nitrophenol are excellent donors.

5. As acceptors, primary alcohols are better than secondary alcohols. The simplest tertiary alcohol tested was not suitable. The presence of an acidic or basic group in the alcohol apparently renders it unsatisfactory as an acceptor. Lactic acid does not accept but its ethyl ester does. Nitration or chlorination of an alcohol does not abolish its ability to accept, nor does the presence of an olefinic linkage. Polyhydroxy alcohols including mannitol serve as acceptors but sugars and inositol do not. Pyridoxal is not converted to codecarboxylase by this method.

6. "Phosphotransferase" action cannot be accounted for by reversal of the phosphate hydrolysis reaction.

7. The effects of pH and the concentration of acceptor and donor on the "phosphotransferase" reaction in the case of nitrophenyl phosphate have been studied, and it was found that the ratio of phosphate transferred to substrate cleaved increases continuously with decreasing pH, while the absolute amount of phosphate transferred passes through an optimum close to that for nitrophenol liberation, but removed from the optimum for phosphate liberation. Increasing the acceptor concentration increases the ratio of phosphate transferred. The absolute amount transferred also increases until the acceptor concentration is great enough to cause inhibition. On the other hand, the ratio is independent of donor concentration.

8. The transphosphorylation between methanol and nitrophenyl phosphate by a citrus juice phosphatase preparation results in the formation of monomethyl phosphate, which has been isolated and identified as the barium salt.

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A UNIFORM MEDIUM FOR DETERMINATION OF AMINO ACIDS WITH VARIOUS MICROORGANISMS*

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The use of lactic acid bacteria for the determination of amino acids which they require is now widespread. The basic techniques used and the various individual methods proposed are very similar, and have been reviewed elsewhere (1–3). These similarities have led some investigators (4, 5) to propose assays of a large number of amino acids under more or less uniform conditions with a single organism. Media used with such general methods have not been optimal for other organisms also useful for assay work. Thus far, however, no single organism has proved suitable for the determination of more than about eight of the amino acids. Lack of an absolute requirement (6, 7), variation in relative activity of optical isomers (8), the now known interrelationships between certain vitamins and some amino acids (9–12), and disturbances resulting from unknown causes combine to make use of a variety of organisms advisable or even necessary when analyses for several different amino acids are required. Use of more than one test organism for the determination of a single amino acid is also valuable as added evidence for or against validity of an assay (1, 2).

An obvious advance in methodology, therefore, would be the development of a single medium which could be made deficient as desired in any single amino acid, and which could be used successfully with any one of a variety of test organisms. This has been the object of the present study. The medium herein proposed has been extensively investigated with respect to its suitability for *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60. For these and four additional species of lactic acid bacteria, it supports acid production equal to or greater than had been previously reported with specialized media for each organism. The single medium with the appropriate amino acid omitted has been employed successfully to determine fourteen different amino acids.

EXPERIMENTAL

Cultures and Inocula—Organisms used were *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, *Leuconostoc mesenteroides* P-60, *Lactobacillus casei*,

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Lactobacillus delbrueckii 5,[1] *Lactobacillus delbrueckii* 3, and *Lactobacillus fermenti* 36. These were carried by biweekly transfer as stab cultures in an enriched basal medium. This was prepared by reducing the concentrations of glucose and sodium citrate shown in the basal medium (Table I) by one-half and adding 0.5 per cent Bacto-tryptone, 0.5 per cent Bacto-yeast extract, and 2 per cent Bacto-agar. All cultures were incubated at 37° until good growth occurred in the stab culture (24 to 48 hours) and were then refrigerated until the next transfer.

For growing inocula, this same medium with agar omitted was employed. This liquid medium was put in tubes in 5 ml. lots, sterilized by autoclaving for 20 minutes at 15 pounds pressure, and kept in the refrigerator. Inocula were prepared by transfer from the stock culture and grown for 8 to 12[2] hours at 37°. The cells were centrifuged and resuspended in a volume of sterile 0.9 per cent sodium chloride solution 5 to 25[3] times greater than that of the medium in which they were grown. 1 drop of suspension was used for each assay tube. Lyophylized cultures, prepared as described by Nymon, Gunsalus, and Gortner (15), were also employed successfully in the later phases of the work. Instead of being used directly as inocula, the resuspended dried cells were transferred to the inoculum medium, incubated for 8 to 12 hours, and used as described above.

Procedure—Assays were carried out in 18 × 150 mm. Pyrex culture tubes, and in a total volume of 2 ml. The samples or standards were added in volumes from 0.2 to 1 ml., water being added to make 1 ml.; 1 ml. of the basal medium, prepared at twice the concentration shown in Table I, was then added. Additions of both water and samples were made with the rapid automatic dispenser of Cannon.[4] Sterilization was accomplished by autoclaving the tubes for 10 minutes at 10 to 12 pounds pressure. A metal cover approximately 3 inches deep and lined with a heavy padding of cotton was placed over each rack of 60 tubes. This cover was removed for approximately 1 minute in a small room previously freed from dust with steam, while 1 drop of inoculum was added to each tube from a sterile pipette.

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[1] Dunn *et al.* (6), because of identical nutritive requirements, have concluded that *Lactobacillus casei* and *L. delbrueckii* 5 are the same organism. Although the nutritive requirements of our culture of *L. casei* correspond to those reported by Dunn, those of *L. delbrueckii* 5 do not. It is possible, therefore, that two different cultures are being used under the latter name.

[2] Inocula grown for 18 to 24 hours may be used at somewhat greater dilution without noticeably affecting the results.

[3] *Streptococcus faecalis* suspensions were diluted approximately 25-fold; suspensions of *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*, and other organisms were diluted from 5- to 10-fold, depending upon the amount of growth. The amount of inoculum used is not a critical factor in determining success of the assay.

[4] Cannon, M. D., in press.

After incubation in a forced air incubator for 60 to 72 hours at 37°, the lactic acid produced was titrated directly in the assay tubes with 0.04 N

TABLE I

Composition of Medium

Component	Quantity per 100 ml.*	Component	Quantity per 100 ml.*
Glucose	2 gm.	Ca pantothenate	100 γ
Sodium citrate (U.S.P.XII)	2 "	Niacin	100 "
" acetate (anhydrous)	0.1 gm.	*p*-Aminobenzoic acid	20 "
NH_4Cl	0.3 "	Biotin	1 "
K_2HPO_4	0.5 "	Folic acid	1 "
Salts C†	2 ml.	DL-Alanine	100 mg.
Adenine sulfate†	1 mg.	DL-Aspartic acid	100 "
Guanine hydrochloride	1 "	L-Glutamic acid‡	100 "
Uracil	1 "	L-Arginine-HCl	20 "
Xanthine†	1 "	L-Lysine-HCl-H_2O	20 "
Thiamine†	100 γ	Other amino acids§	
Riboflavin	100 "	L forms	10 " each
Pyridoxal	20 "	Or DL forms	20 " "

* The quantities indicated are for 100 ml. *final* volume or ten 10 ml. tubes. The first five components are added in dry form each time that a determination is made.

† For convenience, stock solutions containing various related ingredients have been prepared as indicated below, and mixed when making the final medium. Adenine, guanine, and uracil solutions were prepared at concentrations of 1 mg. per ml. with sufficient HCl to keep them in solution. The xanthine at the same concentration was dissolved with the aid of dilute ammonia. The vitamins were combined in a single solution at such concentrations that 1 ml. is used to supply the quantities indicated in Table I. Salts C solution is prepared by dissolving 10 gm. of $MgSO_4 \cdot 7H_2O$, 0.5 gm. of $FeSO_4 \cdot 7H_2O$, 0.5 gm. of NaCl, and 2 gm. of $MnSO_4 \cdot 7H_2O$ and making to 250 ml. (13). The amino acids, exclusive of the one being determined, are prepared in a solution at such concentrations that 25 ml. contain the amounts indicated. Cystine and tyrosine are dissolved first in the minimum quantity of 3 N HCl, then diluted with water and the other amino acids added. The mixture should be heated until complete solution is effected; otherwise crystallization of the ingredients occurs on refrigeration. All solutions are kept under toluene in the cold room and replaced at intervals of 1 month. The medium is adjusted to pH 6.8 to 7.0 before use.

‡ An induction period or lag was encountered in both standards and samples with the glutamic acid determination. It was not corrected by adding 1.6 mg. of L-asparagine per 2 ml. of medium, but was eliminated when the medium was adjusted to an initial pH of 6.0 and a concentrated inoculum used (*cf.* Hac *et al.* (14)). A similar lag in the proline curve was corrected in the same manner.

§ Histidine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, cystine, serine, and glycine.

NaOH. The end-point was determined electrometrically by an adaptation of the technique employed by Pennington *et al.* (16). During titration, the contents of each tube were stirred vigorously with an air stream. The details of this procedure, as applied to volumes of 0.2 ml., are described in the accompanying paper (17). The same technique with correspondingly larger equipment was used in the present studies. Thymol blue and other indicators tried gave unsatisfactory end-points in the presence of the multiple buffers in the medium (Table I), even though the end-point selected corresponded to the inflection point in the titration curve of the buffer mixture. The curve, together with that obtained by electrometric titration of the same buffer mixture, is shown in Fig. 1.

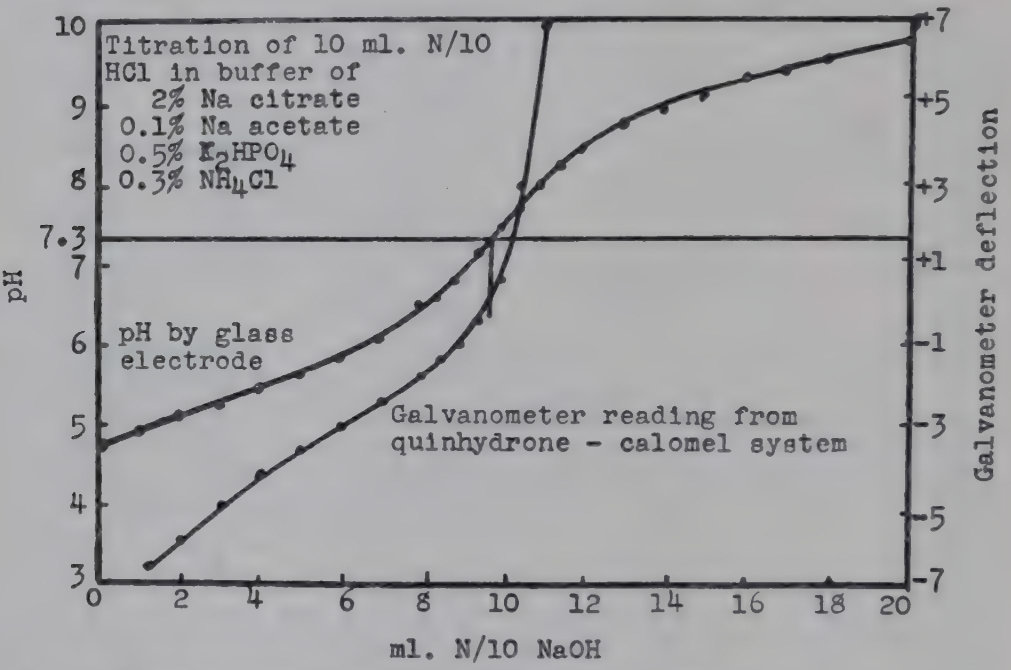

FIG. 1. Curves relating galvanometer deflection to pH of buffered solution

Basal Medium—The composition of the basal medium finally adopted is shown in Table I. In preliminary trials, this medium supported as good or better growth of each of the three assay organisms as did the specialized media then being used in this laboratory (18, 19). These latter media, though essentially alike, differed in minor respects and were representative of improved media used for these organisms by many investigators. The adequacy of the new medium for the various organisms was further tested by running assay curves for leucine in the unchanged medium, and in similar media in which the concentration of single ingredients or a group of related ingredients was varied from a fraction to several times that present in the initial medium. Eleven levels of L-leucine, ranging from 0 to 60 γ per 2 ml., with all points in duplicate, were run to establish each standard curve, and curves were obtained simultaneously with all three test organisms (*Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides*). A superior medium was adjudged to be that variation in which (*a*) the greatest acid production resulted from addition of excess leucine, (*b*) the greatest slope of the standard curve was secured over the assay range, and (*c*) the standard curve was most nearly linear in the assay range. A summary of the results of these investigations is given below.

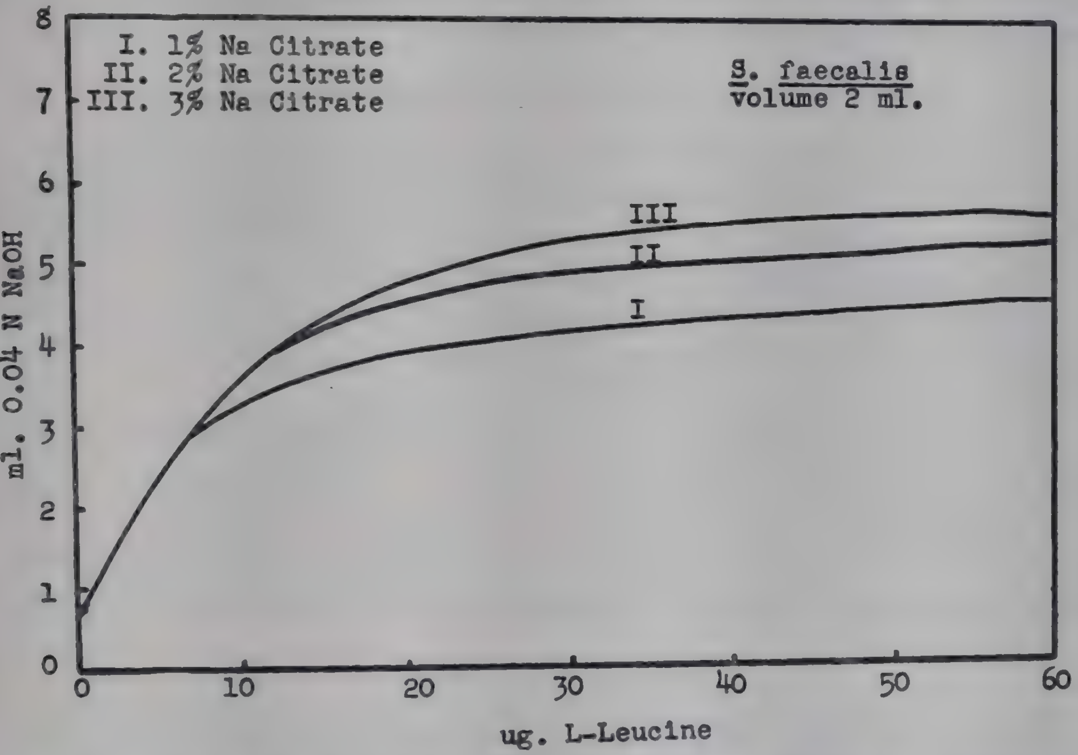

FIG. 2. Effect of sodium citrate concentration on the response of *Streptococcus faecalis* to leucine.

Citrate and Acetate Buffers

The superiority of citrate over acetate buffers in promoting growth and acid production of *Streptococcus faecalis* has been adequately demonstrated (20, 21). Attempts to use citrate buffer for other organisms led to the observations (22) that 2 per cent sodium citrate was toxic, but that the toxicity could be overcome by increasing the concentrations of magnesium and manganese ions in the medium. When such increases in the metallic ion concentrations were made, citrate was an effective buffer for several other lactic acid organisms as well. For *Lactobacillus arabinosus* and *Leuconostoc mesenteroides*, 2 per cent of sodium citrate was superior to 1 or 3 per cent, while for *Streptococcus faecalis* (Fig. 2) 3 per cent gave greater acid production at high concentrations of leucine. Even with this organism, however, the usable portion of the standard curve was not altered by increasing the citrate concentration above 2 per cent. Sodium citrate could be replaced by potassium citrate in equivalent concentrations without altering these results.

Sodium acetate was included in this medium because of its demonstrated growth-stimulating activity for several lactic acid bacteria (23); varying the concentration from 0.05 to 0.3 per cent had no effect on acid production, shapes, or slopes of the standard curves when other buffers were present at the concentrations indicated in Table I.

Ammonium Chloride and Sodium Chloride Concentrations

To eliminate possible disturbing effects of ammonium salts present in protein hydrolysates, ammonium chloride was added to the medium. Variations in concentration from 0 to 1.5 per cent had no effect on the response of *Streptococcus faecalis* or *Leuconostoc mesenteroides* to leucine. At levels of 0.75 per cent and higher, acid production by *Lactobacillus arabinosus* was depressed at all levels of leucine. Ammonium chloride has therefore been added at a lower concentration, 0.3 per cent.

With ammonium chloride at this level, 1 per cent of added sodium chloride was tolerated by *Streptococcus faecalis*. This concentration of salt was slightly inhibitory to the other two organisms but 0.5 per cent was harmless. 10 to 20 mg. of sodium chloride thus represent the maximum allowable amount of this salt which should be added with samples for assay.

Phosphate Concentration

An increase of the concentration of dipotassium phosphate up to 1 per cent resulted in increased acid production by *Streptococcus faecalis* (Fig. 3). *Leuconostoc mesenteroides* P-60 gave smaller increases, while acid production by *Lactobacillus arabinosus* was independent of phosphate concentration between 0.1 and 1 per cent. The assay range for leucine, however, is not lengthened by the addition of amounts of phosphate above 0.5 per cent. This concentration was therefore selected for use in the medium.

Other Inorganic Constituents

Previous results (22) have shown that Salts B at the level usually incorporated do not provide adequate manganese or magnesium to permit optimal growth of assay organisms other than *Streptococcus faecalis* in a medium high in citrate. Salts C (13), which differ from Salts B in that they contain a 4-fold greater concentration of manganese sulfate, have been used. Comparisons of 0.5 per cent Salts B with 0.5 to 4 per cent Salts C were made. Except for *Streptococcus faecalis*, where there was no difference, 0.5 per cent Salts C was superior to 0.5 per cent Salts B. Increasing concentrations of Salts C were beneficial up to 2 per cent; higher levels gave no further improvement (Fig. 4). Salts C, at 2 per cent level, were therefore used in the medium. In separate experiments, addition of a mixture of trace elements (Cr^{+++}, I^-, $B_4O_7^{=}$, Zn^{++}, Cu^{++}, and Co^{++}) to provide 2 γ of each element per 2 ml. had no effect on the response to leucine.

Amino Acid Concentrations

The concentrations of amino acids chosen were considered high, representing in most cases 10 to 20 times the requirement for each in the presence of an excess of the others. The total concentration of the naturally occurring isomers in the medium is 0.38 per cent. Eighteen amino acids were used; hydroxyproline, norleucine, and norvaline were not included, since they have not been reported to affect growth of these organisms in any way at low concentrations. The concentration of the entire mixture of amino acids was first varied from one-half to twice the level indicated in Table I. Slightly diminished acid production by *Streptococcus faecalis* at the lower level was the only effect noted. Glutamic acid, aspartic acid, alanine, arginine, and lysine were varied individually over similar ranges of concentration without effect on the response to leucine of any organism.

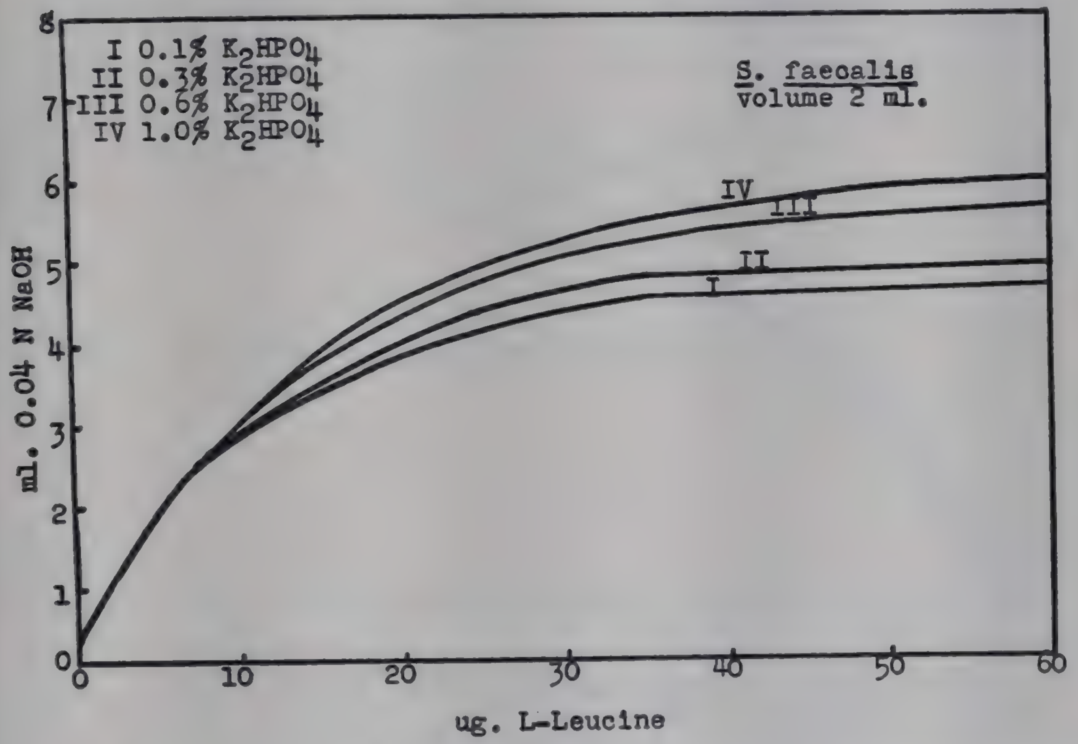

FIG. 3. Effect of phosphate concentration on the response of *Streptococcus faecalis* to leucine.

The originally selected concentrations of amino acids were thus considered adequate.

Glucose Concentration

Glucose concentrations of 1, 2, 3, and 4 per cent were compared. 2 per cent glucose supported acid production equal to that obtained with 3 and 4 per cent for *Streptococcus faecalis* and *Leuconostoc mesenteroides*, but *Lactobacillus arabinosus* showed increased acid production in the upper part of the curve (Fig. 5) with 3 and 4 per cent glucose. While increasing glucose concentration from 1 to 2 per cent greatly increased the usable assay range, no similar increase resulted from higher concentrations of sugar; 2 per cent was therefore selected for use in the medium.

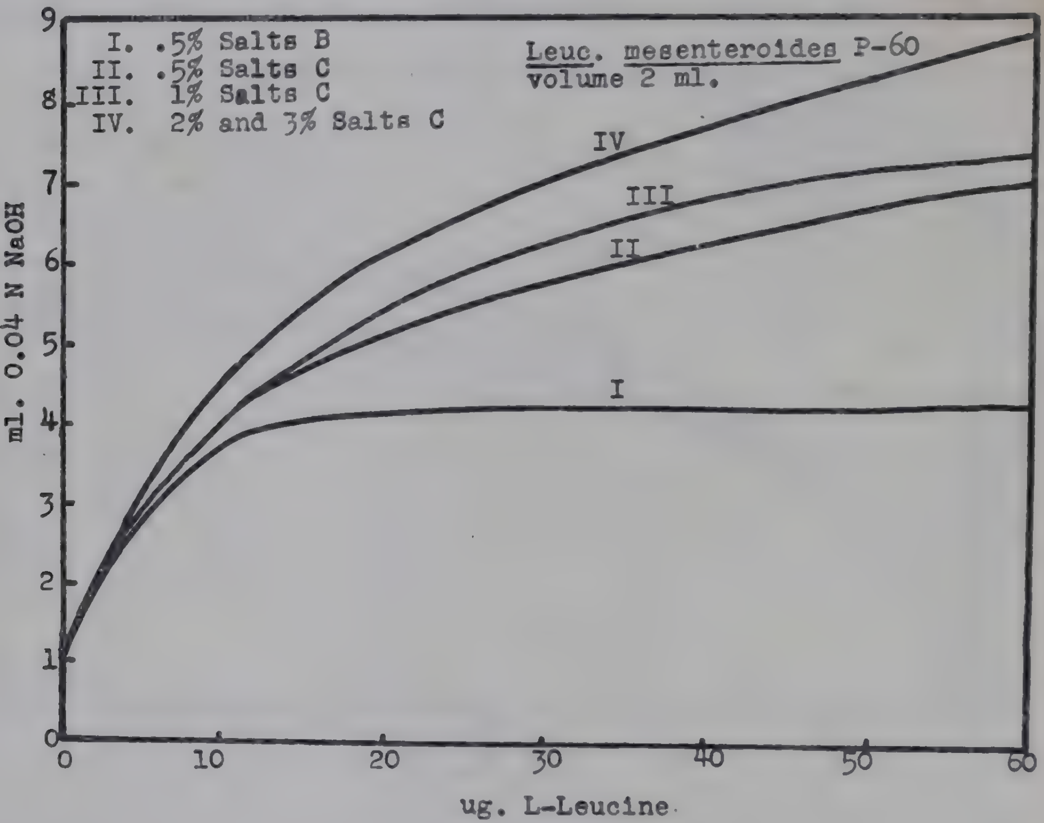

FIG. 4. Effect of concentrations of inorganic salts on the response of *Leuconostoc mesenteroides* P-60 to leucine.

It should be noted that the curves obtained with 1 or with 2 per cent of sugar are superimposable over the lower part of the assay curve. If one wishes to work within this range, there is no evidence that any advantage (*i.e.*, greater accuracy or higher precision) would result from the use of sugar concentrations above 1 per cent.

Vitamin Concentrations

The vitamins were added at relatively high concentrations. Pyridoxal, the form of vitamin B_6 available to each of these microorganisms, was used. Concentrations of the entire vitamin mixture equal to one-eighth and one-fourth that shown in Table I were inferior, especially for *Leuconostoc mesenteroides* and *Streptococcus faecalis.* Levels from one-half to twice that adopted gave identical standard curves.

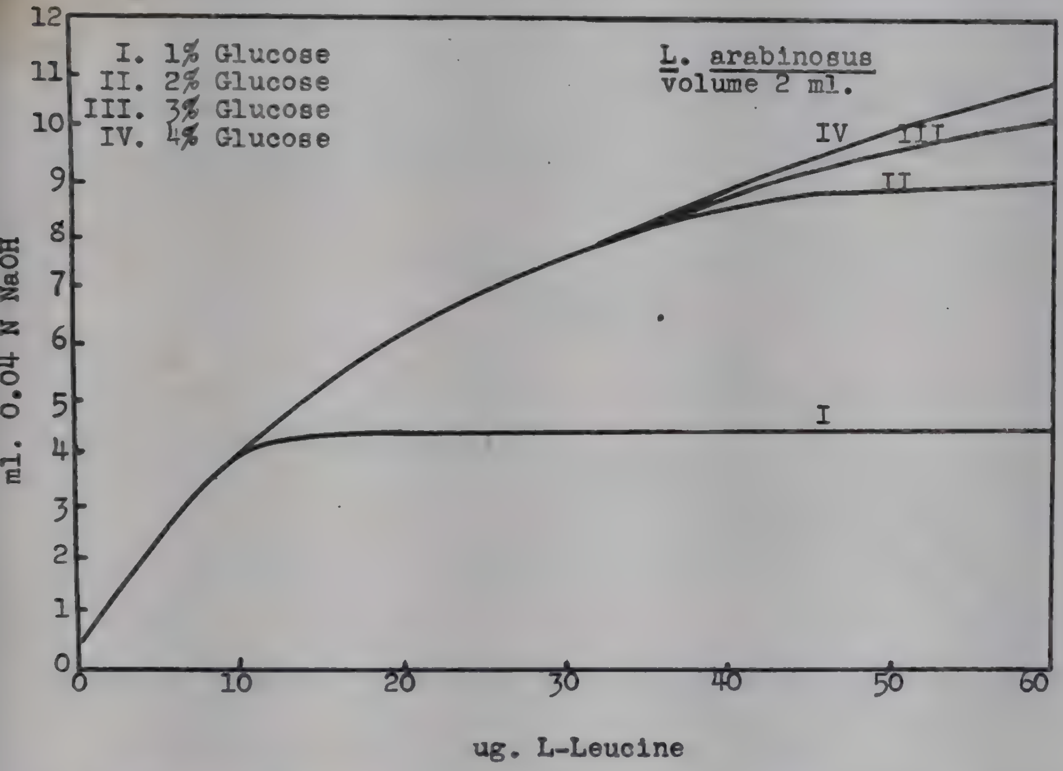

FIG. 5. Effect of glucose concentration on the response of *Lactobacillus arabinosus* to leucine.

Purine and Pyrimidine Bases

Omission of the purine and pyrimidine bases from the medium resulted in an induction period in the leucine response curve obtained with *Leuconostoc mesenteroides.* Other organisms were unaffected. No improvement in response curves with any organism resulted from varying the concentration of this supplement from one-half to twice that adopted.

Use of Medium with Other Test Organisms

The adequacy of the assay medium for *Lactobacillus arabinosus, Streptococcus faecalis,* and *Leuconostoc mesenteroides* having been proved, it was next compared with previously described media for its ability to support growth and acid production of several other lactic acid bacteria. The results of a comparison for *Lactobacillus delbrueckii* 5 with the medium of Stokes *et al.* (4) are shown in Fig. 6. The maximum acid production and the shape and slope of the standard curves differ markedly.

Similar data for *Lactobacillus casei* grown on the medium of McMahan and Snell (24) are shown in Fig. 7. Here the curves were identical in the lower portion, but the new medium gave a greatly extended assay range when the titrimetric method was used, in part because of the increased amounts of sugar and buffer present. *L. fermenti* gave acid production on this medium comparable to that recently reported with a specialized medium by Dunn *et al.* (6). *L. delbrueckii* 3 likewise grew well, giving approximately 90 per cent of the theoretical acid production (considered to be 11 ml. of 0.04 N acid per 2 ml. of medium) in the complete medium. It was concluded that the medium can be employed unchanged for most of the lactic acid bacteria hitherto used for assay of amino acids.

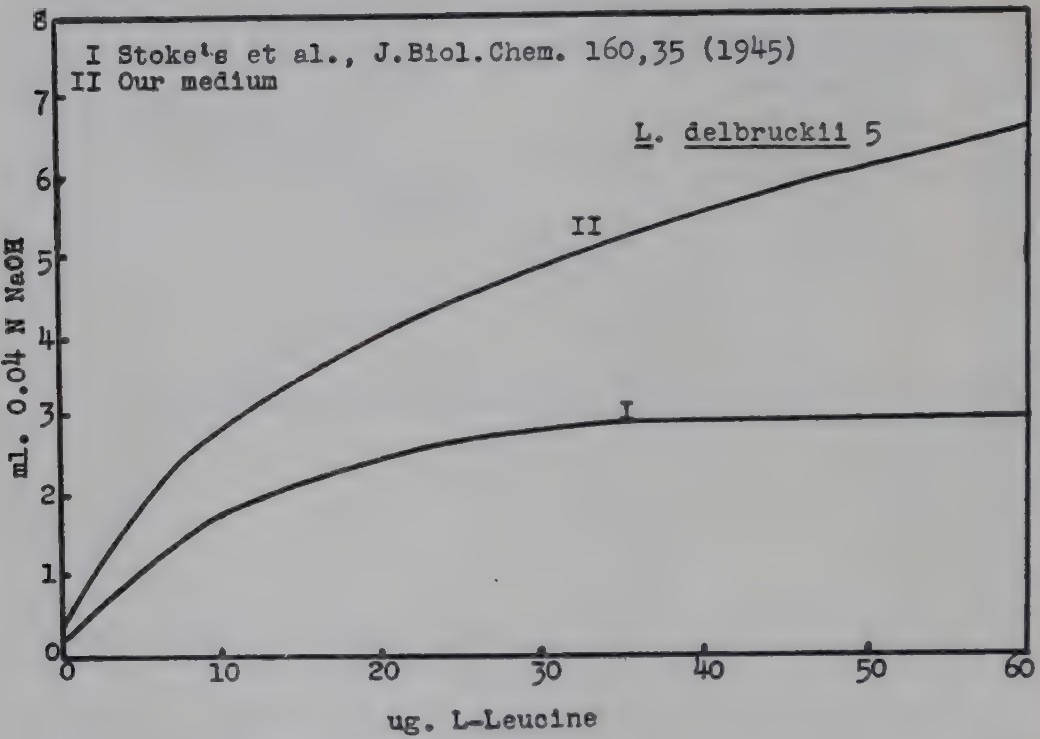

FIG. 6. Comparative response of *Lactobacillus delbrueckii* 5 to leucine in two assay media.

Analyses of Protein Hydrolysates—Four purified proteins were analyzed a number of times for fourteen different amino acids, this medium and a number of test organisms being employed. Hydrolysis was accomplished by autoclaving 0.5 gm. of the protein in sealed Pyrex tubes with 20 ml. of 3 N hydrochloric acid for 5 hours at 15 pounds pressure. Variation of the hydrolysis procedure for casein from 2 N hydrochloric acid for 5 hours to 3 N hydrochloric acid for 10 hours did not alter the results significantly. For tryptophan analyses, both enzymatic and alkaline hydrolysates were employed. The alkaline hydrolysates were prepared by autoclaving 0.5 gm. of protein with 10 ml. of 5 N sodium hydroxide in a sealed Pyrex tube for 15 hours at 15 pounds pressure. Complete racemization was assumed (25). Enzyme digestion (26) was accomplished by shaking 50 ml. of an unbuffered suspension containing 200 mg. of protein, 20 mg. of Merck's pancreatin, 4 mg. of Wilson's hog intestinal mucosa, and a little toluene for 3 days.

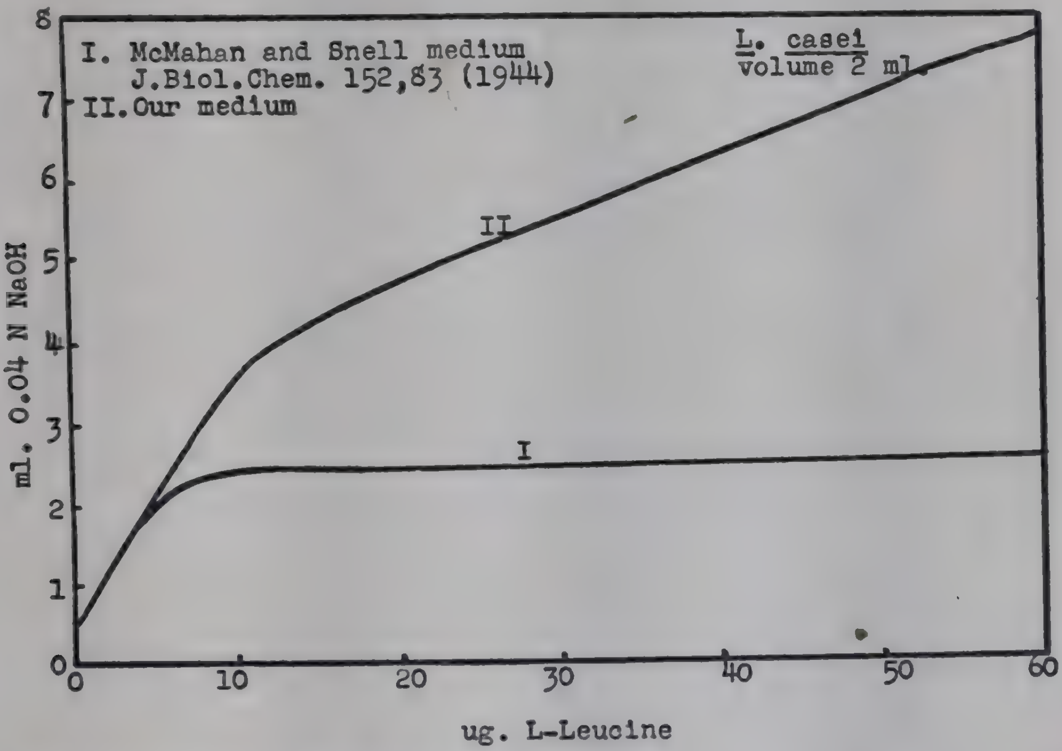

FIG. 7. Comparative response of *Lactobacillus casei* to leucine in two assay media

Each determination was made with three to six tubes at each of six levels of the amino acid standard and with three tubes at each of five levels of the samples (0.2, 0.4, 0.6, 0.8, and 1 ml.). In most cases, analyses were conducted simultaneously for at least six amino acids.

Such extensive assays were made primarily to test the applicability of the new medium to the assay of each of the several amino acids for which test organisms are available. The results, though apparently comparable in precision to those reported by previous workers, are not felt to represent the maximum precision attainable in microbiological assays with this or other media.

The results of these analyses are shown in Table II and are compared with the range of values from the literature found by microbiological assay

TABLE II

*Amino Acid Composition of Purified Proteins**

Amino acid†	Casein		Bovine serum albumin		Edestin		Colostrum pseudoglobulin	
	Gm. per 100 gm.	Literature values	Gm. per 100 gm.	Literature values	Gm. per 100 gm.	Literature values	Gm. per 100 gm.	Literature values
Arginine	3.78 ± 0.65^{5}‡	3.8 ± 0.4^{6}‡	6.2 ± 0.5^{3}	6.1 (27) 6.2 (28)	17.4 ± 0.1^{2}	16.7 (29)	5.20 ± 0.16^{3}	5.6 (30)
Aspartic acid	7.4 ± 0.03^{2}	6.9 ± 0.8^{3}	11.1 ± 0.16^{3}	10.6 (28)	13.4 ± 0.5^{3}		9.3 ± 0.3^{4}	9.4 (30)
Glutamic "	21.7 ± 1.7^{6}	21.6 ± 0.8^{7}	16.6 ± 0.9^{5}	16.9 (28) 18.0 (27)	19.4 ± 1.5^{5}	2.13 (14) 19.1 (31)	10.7 ± 1.5^{4}	12.3 (30)
Histidine								
S. faecalis	2.82 ± 0.1^{4}		3.62 ± 0.2^{3}	3.80 (28)	2.61 ± 0.04^{2}	2.62 (29)	2.22 ± 0.16^{4}	2.3 (30)
L. mesenteroides	3.03 ± 0.24^{4}	3.06 ± 1.0^{7}	3.7 ± 0.17^{2}	4.1 (27)	2.66 ± 0.01^{2}		1.92 ± 0.17^{4}	
Isoleucine	7.6 ± 0.35^{9}	6.4 ± 1.6^{5}	2.97 ± 0.2^{2}	3.3 (27) 2.9 (28)	6.47 ± 0.02^{2}	6.21 (29)	4.1 ± 0.11^{3}	4.2 (30)
Leucine	10.25 ± 0.3^{8}	9.6 ± 2.4^{8}	11.8 ± 0.6^{2}	11.3 (27) 13.7 (28)	7.5 ± 0.05^{2}	7.43 (32) 7.4 (28)	8.3^{1}	8.5 (30) 8.5 (36)
Lysine	8.07 ± 0.3^{3}	7.76 ± 1.1^{8}	10.3 ± 1.1^{3}	12.4 (28) 12.3 (27)	2.09 ± 0.09^{3}	2.49 (29)	6.13 ± 0.42^{4}	6.1 (30)
Methionine	2.69 ± 0.26^{6}	2.85 ± 0.21^{6}	0.86 ± 0.04^{4}	0.81, 0.77 (33) 0.81 (28)	2.07 ± 0.13^{4}	2.35 (35) 2.12 (34) 2.22 (29)	1.1 ± 0.18^{5}	1.3 (30)
Phenylalanine	5.45 ± 0.34^{8}	5.36 ± 0.5^{6}	6.48 ± 0.22^{2}	6.4 (27) 6.2 (28)	5.22 ± 0.07^{2}	5.45 (32)	4.25 ± 0.32^{3}	3.68 (36) 3.9 (30)
Proline	11.6 ± 0.7^{6}	11.2^{1}	5.1 ± 0.3^{2}	5.6 (28)	4.6 ± 0.24^{4}			10.0 (30)
Threonine	4.28 ± 0.28^{10}	4.2 ± 0.4^{6}	6.2 ± 0.2^{2}	6.5 (28) 6.3 (27)	3.7 ± 0.5^{4}	3.14 (29)	10.2 ± 0.8^{4}	9.0 (30)
Tyrosine	6.2 ± 0.7^{6}	5.8 ± 0.7^{4}	4.3 ± 0.4^{2}	5.2 (27) 5.49 (28) 5.3 (37)	3.7 ± 0.2^{2}			6.7 (30)

Valine	7.15 ± 0.4^{11}	6.67 ± 1.1^{8}	6.6 ± 0.04^{2}	5.4 (27) 6.5 (28)	6.6 ± 0.1^{2}	6.63 (32)	9.3 ± 1.0^{3}	9.1 (36) 8.7 (30)
Tryptophan								
Hydrolyzed by enzymes	1.28^{1}		0.41^{1}		0.86^{1}		3.1^{1}	3.2 (30)
Hydrolyzed by NaOH	1.24^{1}	1.18 ± 0.2^{7}	0.49^{1}		1.02^{1}	1.24 (32)	2.2^{1}	2.27 (36)

* All values are expressed as gm. of amino acid per 100 gm. of dried ash-free protein. The casein was prepared from skim milk according to the procedure of Cohn and Hendry (38). It was washed by resuspending the protein twice in 95 per cent ethanol, once in absolute ethanol, and three times in ether; N = 15.2 per cent, ash = 0.5 per cent (dry basis). Bovine serum albumin was kindly supplied by Dr. L. L. Lachat of the Armour Laboratories, Chicago; N = 15.9 per cent, ash = 0.2 per cent (dry basis). Edestin was prepared from hemp seed by the method of Osborne (39), and was recrystallized four times from sodium chloride solution; N = 18.7 per cent, ash = 0.55 per cent (dry basis). Colostrum pseudoglobulin was kindly supplied by Mr. R. G. Hansen of this department; N = 16.0 per cent, ash negligible (30). The amino acids used as standards were dried *in vacuo* at 30° and kept *in vacuo* in a desiccator containing anhydrous calcium chloride. DL forms of isoleucine, valine, methionine, threonine, and phenylalanine were employed; the natural isomers of the others were used. Products obtained from three to five sources were compared for microbiological activity. With one exception (isoleucine), the activity of the various samples checked within the limits of experimental error. Merck's DL-isoleucine was used as the standard in this instance. DL-Methionine was employed throughout these studies as a standard, though unpublished data from this laboratory indicate that D-methionine has measurable activity for *Streptococcus faecalis* in the presence of L-methionine. Hence the values reported here may be slightly low.

† *Lactobacillus arabinosus* was used for glutamic acid, leucine, phenylalanine, valine, and tryptophan, *Streptococcus faecalis* for arginine, histidine, methionine, and threonine, *Leuconostoc mesenteroides* for aspartic acid, histidine, lysine, proline, tyrosine, and isoleucine. Severe drift in assay values was encountered when *L. arabinosus* was employed for isoleucine determination; *Leuconostoc mesenteroides* and four other organisms gave values for isoleucine which agreed and showed no noticeable drift. Recent results (Sirny *et al.*, unpublished) indicate that *L. delbrueckii* 3 is more suitable for arginine assay than is *Streptococcus faecalis*.

‡ Superscripts indicate the number of determinations made or the number of values cited as averages from literature. In all columns the variation indicates the extreme values found.

of these same proteins. In view of the small number of analyses made in some cases, the range of values is given, and the data are not treated statistically.

The data represent the results of separate analyses of a number of different acid hydrolysates of each protein (*e.g.*, eight for casein). A solution of amino acids, compounded to contain roughly the relative concentration of each amino acid required by the various test organisms, was analyzed each time. With very few exceptions, the amount of each amino acid found by assay of this mixture agreed to within ±5 per cent with the amount added, and the agreement was usually much better than this.

SUMMARY

1. A uniform medium has been developed for the microbiological determination of amino acids with a number of the most widely used lactic acid bacteria. High acid production, low blanks, and satisfactory standard curves for each of fourteen different amino acids were obtained.
2. The concentrations of single or closely related components of the medium were varied over wide ranges and approximately optimal concentrations for each selected for use in the medium.
3. Hydrolysates of purified casein, edestin, bovine serum albumin, and colostrum pseudoglobulin were analyzed for fourteen amino acids. Reproducible values, in most cases comparable in magnitude and variation with those reported from other laboratories by microbiological methods, were obtained.

Addendum—Since the submission of this paper, the above medium and method have been found suitable for assay of glycine. *Leuconostoc mesenteroides* is used as the test organism. For this purpose, it is ordinarily necessary to recrystallize the DL-alanine used in the medium, since this amino acid, as obtained commercially, is sometimes contaminated with glycine. A sample of Merck's DL-alanine, for example, was found to contain 0.6 per cent of glycine by microbiological assay. This was eliminated by one recrystallization.

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A MICROMETHOD FOR THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS*

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A rapid, routine method for determining amino acids in tissues, enzymes, hormones, viruses, and other natural materials available only in small quantities would have wide application. The microbiological technique should be well suited to such a method because (*a*) it can be applied for the majority of the amino acids with only slight variations in procedure and because (*b*) the method is sensitive at conventional volumes and would be extremely sensitive if adapted to small volumes. Micro adaptations of microbiological assays for vitamins have been described by Pennington *et al.* (1) and by Lowry and Bessey (2) in which 0.2 ml. of medium was used. The latter workers, employing *Lactobacillus casei* for riboflavin determinations (3), found that the chief difference between the conventional method and the micro adaptation was the need for incubation of the small tubes in an atmosphere of carbon dioxide saturated with water and the addition, prior to inoculation, of a solution of cysteine sterilized by filtration.

A similar micromethod for the determination of amino acids is described below which has been applied successfully in analyzing hydrolysates of purified proteins. With this method, at least fourteen amino acids can be determined in 10 to 20 mg. of a protein of average composition.

Procedure

The medium used, the preparation of inocula, and the general procedure were those previously described (4). The only changes in technique were those necessitated by the reduction in final volume from 2 to 0.2 ml.

Standard $\frac{3}{8} \times 3$ inch lipless culture tubes were held in racks 9×14 cm. made from sheet copper, each with a capacity of 60 tubes. After the sample or standard solutions were placed in the assay tubes at levels of 0, 0.02, 0.04, 0.06, 0.08, and 0.1 ml., water was added to bring the volume to 0.1 ml. in all the tubes. Suitable special pipettes for these volumes have been described elsewhere (1, 2, 5). The tubes were then closed with plugs or with a suitable cover similar to that previously described (4) and autoclaved for

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10 minutes at 10 pounds pressure. Double strength basal medium was sterilized in the same manner in a plugged flask. When the medium had cooled, it was inoculated with 1 ml. of inoculum suspension per 20 ml. of medium. 0.1 ml. of the inoculated medium was pipetted aseptically into each assay tube. This should be done in a sterilized hood or dust-free room, since flaming of the tubes is impracticable.

The tubes were incubated for 60 to 72 hours in a covered water bath at 37°. Under these conditions evaporation was negligible. In the circulating air incubator, evaporation was too great and variable to permit reliable assays. The lactic acid was titrated electrometrically directly in the assay tubes with 0.04 N sodium hydroxide from a micro burette. Calculations, etc., were made in the same manner as when assays are conducted on the usual scale.

Mechanical Aids Employed

In the studies described here, special mechanical aids were employed for pipetting and titrating. While these are not necessary and do not increase the precision of the method, they greatly facilitate the determinations.

Measurements of volumes were made with the Cannon dispenser.[1] The liquid being measured passes through a small orifice under fixed pressure for controlled periods of time to give the desired volumes. Any desired sequence of volumes can be dispensed. A special solenoid valve (*5*, Fig. 1, *A*), 2 × 1 × 1 inches, essentially similar to that described by Cannon,[1] was constructed from a small 12 volt, D.C. solenoid. The blade of this solenoid valve is thrust to the right by a spring when the circuit is open, compressing the rubber tubing, *7*, against a fixed bar, *8*, thus interrupting the flow. The dispensing tip, *10*, was prepared by drawing out 3 mm. glass tubing in a flame to the point of complete constriction, and then cutting the tube near the termination of the constriction and grinding the cut surface back toward the lumen of the tube until the resulting orifice reached the appropriate diameter for the volume to be dispensed (less than 0.1 mm. for the studies described here).

The vessels used are shown in Fig. 1; vessel *A* was used to contain the medium and vessel *B* for sample and standard solutions. The liquids are dispensed from the tube, *4* or *12*, downward into the assay tubes under fixed air pressure exerted above (*1*, Fig. 1, *A*). Approximately forty measurements of any desired volume or sequence of volumes from 0.02 to 0.1 ml. can be made in 1 minute. The accuracy is ±1 per cent at 0.1 ml. Although individual weighings of liquid dispensed by the 0.02 ml. segment were not made, five such dispensings weighed 0.1 gm. ± 0.001 gm.

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[1] Cannon, M. D., in press.

The sample vessel (Fig. 1, *B*) has a capacity of approximately 5 ml. The ground glass joint, *13*, permits rapid disassembly for washing the tube, *12*, and rinsing with the next solution to be dispensed. A number of such vessels can be used in turn, the inner joint, *13*, remaining in position in the rubber tubing of the dispensing assembly, as shown in Fig. 1, *A*.

The constriction in the air inlet tube (*2*, Fig. 1, *A*) holds a cotton filter in place so that aseptic delivery of solutions (*e.g.*, inoculated media) is readily accomplished. Each medium tube, *4*, is fitted with rubber tubing approximately 2 inches long with a tapered glass rod closing the open end in place of the tip, *10*. Each vessel is also fitted with a brass bar, *8*, held in position by attached copper wire, *9*, with spirals through which the rubber tubing, *7*, is threaded. This entire assembly, detached from the solenoid valve, *5*, is autoclaved with the basal medium required. As many as fourteen of these vessels have been autoclaved simultaneously, supported by spring clips around the upper joint, *3*, attached to a strip of wood on a ring stand.

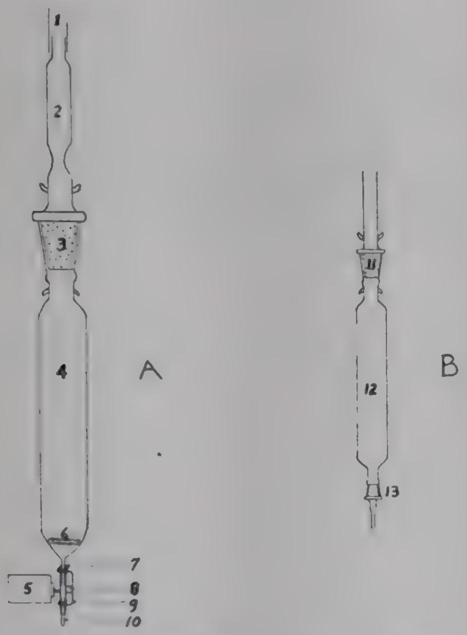

FIG. 1. Dispensing vessels and solenoid valve assembly for use with the Cannon dispenser. *1*, inlet for air under controlled pressure, *2*, cotton air filter, *3*, 14/35 ground glass joint, *4*, 25 mm. Pyrex tube 10 cm. in length, *5*, 12 volt D.C. solenoid, *6*, glass-encased nail, *7*, rubber tubing 1/16 × 1/32 inch, *8*, brass bar 5 × 5 × 35 mm., *9*, No. 18 copper wire, *10*, dispensing tip, *11*, 10/30 ground glass joint, *12*, 12 mm. Pyrex tube 7 cm. in length, and *13*, 5/20 ground glass joint.

When the medium has cooled, the inner joint of the air inlet, *3*, is removed, the outer joint flamed, and 1 drop of inoculum, prepared as previously described (4), is added for each ml. of sterile, double strength basal medium. The air inlet tube is then replaced. Stirring is accomplished by moving a glass-encased nail, *6*, up and down in the medium with the aid of a small horseshoe magnet. When the vessel is clamped into position with the bar, *8*, attached rigidly to the body of the solenoid, the valve closes on the tubing, *7*, and the glass rod is replaced by the dispensing tip, *10*, which has been separately sterilized in mercuric chloride solution. The tip and tubing are rinsed well with inoculated medium; then 0.1 ml. is dispensed into each tube.

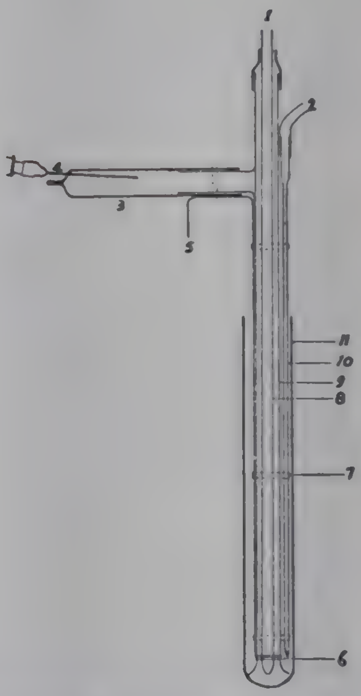

Fig. 2. Assembly for electrometric titration in culture tubes. *1*, 3 mm. glass tubing admits the alkali; *2*, rubber tubing connecting capillary salt bridge, *10*, to calomel half-cell; *3*, rubber policeman over side arm; *4*, hypodermic needle admits a suitable stream of CO_2-free air for stirring (air passes between the walls of concentric tubes, *8* (diameter 3 mm.) and *9* (5 to 6 mm.)); *5*, Pt wire from the terminal of the galvanometer to the coil of Pt, *6*, wrapped around the tube, *9*, to form an electrode; *7*, small rubber bands to hold the Pt wire, *5*, and the salt bridge, *10*, in position; *11*, 3/8 × 3 inch culture tube.

Extreme care must be exercised to prevent solid materials such as chips of glass, cotton fibers, or sediment in the medium, standards, or samples from clogging the orifice of the tip. To prevent this, all samples, standards, water, and media to be dispensed are carefully filtered through No. 42 Whatman paper. Ground glass joints were left dry, since stop-cock lubricants also clogged the orifice. By the technique just described, 1500 tubes involving 4500 pipettings can be set up in an 8 hour day, provided the samples are prepared and diluted in advance.

Since the titration procedure has not been previously described and has proved very satisfactory, both for 0.2 ml. and 2 ml. volumes (4), it will be outlined here in some detail.

The apparatus used is shown in Fig. 2. It can be readily assembled from available laboratory equipment. Its essential parts are a quinhydrone electrode connected through a capillary salt bridge *2* and *10*, to a calomel half-cell (1 M KCl) with the external circuit closed through an inexpensive galvanometer (Leeds and Northrup portable pointer type, Cenco No. 82150D or 82155D, 0.5 microampere per scale division or equivalent).

The potential of such a cell is 0 at approximately pH 7.1 and the galvanometer can be readily adjusted to give no deflection at the desired end-point. In this case (4) the end-point chosen was pH 7.3. At this end-point, the addition of 0.005 ml. of 0.04 N sodium hydroxide to 0.2 ml. of the culture medium results in a deflection of one-half scale division on the galvanometer.

Stirring is accomplished by passing carbon dioxide-free air under pressure through a No. 27 or No. 28 hypodermic needle, *4*, into a side arm through the wall of a rubber policeman, *3*. The air passes between the walls of the concentric tubes, *8* and *9*, and bubbles up through the solution.[2] The platinum wire, *5* and *6*, which serves as the inert conductor for the quinhydrone electrode, is wound around the outer tube, *9*, three to five times. Small rubber bands, *7*, hold the salt bridge and platinum wire in position. The outer tube is flanged at the tip to protect the salt bridge and to give more effective stirring. The salt bridge, *10*, should be made as large as possible, with the flow of KCl solution impeded adequately by shrinking of the tip in a flame around a few fibers of fibrous asbestos. If the diameter of the capillary is too small, the sensitivity is greatly reduced.

Although this equipment can be used with a micro burette, most of the work described here was done by the technique described by Cannon.[3] In this procedure, the dispenser used for filling the tubes was also used for titrating. Instead of measuring volumes, the instrument is used in such a way that it records on an automatic counter the length of time that alkali is allowed to flow through a small orifice at constant pressure. Repeated checks indicate that the probable titration error is between 1 and 2 per cent; *i.e.*, 0.005 to 0.01 ml. in a total titration of 0.5 ml. A similar percentage error was found for the 2 ml. titrations (4).

Before titration, quinhydrone is added to each tube as 1 drop of a water suspension of such concentration that a slight amount remains undissolved when the titration is complete. With some cells, the galvanometer deflection at the end-point may change slightly during the first few minutes of use. For this reason a buffer solution which contains the major components of the basal medium, or a portion of the basal medium itself, adjusted to the desired end-point, should be checked after each five to ten tubes to ascertain the corrected galvanometer deflection until it requires no further adjustment, after several such checks. Usually no adjustment is needed after the first tube. With practice, one can titrate at least two tubes per minute with less effort and less chance of reading error than in using a burette and indicators.

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[2] A similar device for stirring and titrating the contents of assay tubes has been used with burette and indicators by Dr. F. M. Strong and associates in this laboratory.

[3] Cannon, M. D., to be published.

Results

A preliminary study showed that the test organisms being employed, *i.e. Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, *Leuconostoc mesenteroides* P-60, and *Lactobacillus delbrueckii* 3, would grow and produce acid satisfactorily in these small volumes. Under the conditions used, incubation in an atmosphere of carbon dioxide and addition of cysteine, as recommended (2) with *L. casei* in the microdetermination of riboflavin, were not necessary when *L. arabinosus* was used and leucine was the limiting amino acid. Standard curves obtained with and without these precautions, in all four possible combinations, were superimposable. Incubation in air with no added cysteine was also employed with the other test organisms without noticeable difficulties, due to this cause.

To test the applicability of the microprocedure, a series of parallel assays were made with the macroprocedure (2 ml. volumes) previously described (4) and the microprocedure (0.2 ml. volumes) described above. The same hydrolysates, basal media, inocula, etc., were used in both cases. Samples and standards were set up in triplicate at each of five levels.

The shapes, slopes, and maxima of the standard curves obtained were very similar at the 2 volumes. On the micro scale, titrations thus range from 0 to almost 1.0 ml. of 0.04 N alkali. No consistent variations which could be attributed to the differences in volume were noted.

The results of several parallel analyses of four purified proteins for three amino acids are shown in Table I. Similar parallel assays at the 2 volumes were conducted for ten other amino acids. The average values are given in Table II. The variations among individual assays were comparable to that shown in Table I. In general, variations were somewhat greater for the 0.2 ml. than for the 2 ml. determinations. Results of analyses of a known mixture of amino acids by the micromethod were also somewhat more variable than those obtained with the 2 ml. assays. With few exceptions, such analyses gave values between 90 and 110 per

TABLE I

Leucine, Valine, and Threonine Values for Purified Proteins As Determined by Parallel Macro and Micro Assays

The results are given in gm. per 100 gm. of protein.*

Protein	Leucine		Valine		Threonine	
	2 ml.	0.2 ml.	2 ml.	0.2 ml.	2 ml.	0.2 ml.
Casein	10.1	9.5	7.2	7.2	4.2	4.2
	10.2	10.6	7.3	6.8	4.1	4.0
	10.3	9.9	7.1	6.5	4.2	4.5
	10.1	10.7	7.2	7.0	4.1	4.3
Bovine serum albumin	11.3	11.5	6.6	6.7	6.3	6.5
			6.6	6.3	6.0	6.4
Edestin	7.4	7.2	6.5	6.3	3.4	3.8
Colostrum pseudoglobulin	8.3	8.5	9.4	10.2	9.2	10.1
			10.2	9.1	9.9	9.8

* Same proteins as described previously (4). The values are expressed on an ash- and moisture-free basis.

TABLE II

*Amino Acid Composition of Purified Proteins As Determined by Macromethod and Micromethod**

Amino acid	Casein		Bovine serum albumin		Edestin		Colostrum pseudoglobulin	
	2 ml.	0.2 ml.	2 ml.	0.2 ml.	2 ml.	0.2 ml.	2 ml.	0.2 ml.
Arginine	3.8^5	4.1^5	6.2^3	6.2^4	17.4^2	18.3^2	5.2^3	5.1^3
Aspartic acid	7.4^2	7.5^2	11.1^3	10.6^2	13.4^3	12.6^2	9.3^4	9.2^3
Glutamic "	21.7^3	22.5^2	16.6^5	17.0^2	19.4^5	19.7^1	10.7^4	12.6^2
Histidine (*Streptococcus faecalis*)	2.8^4	3.0^4	3.6^3	3.8^3	2.6^2	3.0^2	2.2^4	2.3^3
Histidine (*Leuconostoc mesenteroides*)	3.0^4	3.2^4	3.7^2	4.1^2	2.7^2	3.5^2	1.9^4	2.0^2
Isoleucine	7.6^9	8.3^1	3.0^2	2.8^1	6.5^2	6.9^1	4.1^3	3.9^1
Leucine	10.3^8	10.1^5	11.8^2	10.1^3	7.5^2	7.2^1	8.3^1	8.5^1
Lysine	8.1^3	8.9^3	10.3^3	10.2^1	2.1^3	2.4^2	6.1^4	6.9^3
Methionine	2.7^6	2.7^5	0.86^4	0.73^3	2.1^4	2.1^2	1.1^5	1.1^4
Phenylalanine	5.5^8	5.4^9	6.5^2	6.8^2	5.2^2	6.2^1	4.3^3	4.5^3
Proline	11.6^6	12.0^2						
Threonine	4.3^{10}	4.3^4	6.2^2	6.4^3	3.7^4	3.9^2	10.2^4	10.0^3
Tyrosine	6.2^6	6.6^3	4.3^2	4.2^1	3.7^2	3.7^1		
Valine	7.2^{11}	6.8^8	6.6^2	6.5^3	6.6^2	6.2^1	9.3^3	9.3^3

* The values given are averages of the number of determinations shown in superscripts. All are expressed as gm. of amino acid per 100 gm. of dry, ash-free protein.

cent of the amino acids added, as compared with the 95 to 105 per cent obtained when the larger volumes were used (4). With further refinement, greater accuracy on the micro scale should be attainable.

SUMMARY

A micro adaptation of an existing microbiological procedure (4) for determining amino acids is described which utilizes 0.2 ml. total volume. Parallel assays for thirteen amino acids in protein hydrolysates by the macroprocedure and microprocedure indicate that, in most cases, the precision of the micro adaptation approaches that of the assay conducted at customary volumes. The method does not involve changes in the procedure other than those inherent in the change to a smaller volume.

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THE UTILIZATION OF D-AMINO ACIDS BY MAN*

VIII. TRYPTOPHAN AND ACETYLTRYPTOPHAN

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In a previous publication (1) we expressed the opinion that, with the exception of a 5 per cent urinary loss, all of the orally administered acetyl-DL-tryptophan may be available to man. Subsequently Luck, Boyer, and Hall (2) found that 70 to 83 per cent of intravenously administered acetyl-DL-tryptophan was excreted unchanged in the urine within 6 hours of the injection. These findings led them to the conclusion that intravenously administered acetyl-L- and acetyl-D-tryptophan are not utilized by man.

The divergent conclusions reached in the two studies may be the result of (*a*) differences in chemical characteristics of the assay methods employed or (*b*) differences arising from the routes of administration of the test substances. The wide-spread practical and fundamental implications of the latter possibility prompted us to reinvestigate the fate of orally administered acetyltryptophan by measurements of urinary metabolites in the adult and nitrogen balance and body weight changes in the infant. Our original contention that orally administered acetyl-DL-tryptophan may be completely utilized by man received additional support from the failure to find by the colorimetric methods of Luck and associates any increase of tryptophan values in the urine of subjects fed acetyl-L- or acetyl-DL-tryptophan. Furthermore no trace of acetyltryptophan could be detected by application of their isolation procedures to these specimens directly or to 5-fold concentrates of the urines. Feeding tryptophan-deficient diets supplemented in turn for 4 day periods with L- or DL-tryptophan and acetyl-L- or acetyl-DL-tryptophan to infants disclosed that subnormal growth and N retention were induced in all three subjects only by the diet reinforced with DL-tryptophan. This additional evidence of the utilization of acetyl-L- and acetyl DL-tryptophan indicates beyond a doubt that the metabolic fate of these substances may be drastically affected by the manner of administration.

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EXPERIMENTAL

Commercially available L- and DL-tryptophan (Merck), containing 13.5 and 13.6 per cent N respectively, were employed in these experiments. The necessary acetyl derivatives of the compounds were obtained in good yields by the method of du Vigneaud and Sealock (3) and the nitrogen content and specific rotation of the products found to be in good accord with the accepted values.

Excretion Experiments—As in the previous studies adult subjects were given 0.01 mole (2.46 gm.) of acetyl-L- or acetyl-DL-tryptophan in 240 cc. of water. The urines were collected and pooled for the succeeding 24 hours and analyzed subsequent to adjustment of the pH to 3 with dilute HCl. It is apparent from the typical data shown in Table I that the ingestion of either isomer of acetyltryptophan did not increase the titer of aromatic metabolites of the urines above the control levels as measured by the procedures described by Luck and his associates. This finding and the fact that acetyltryptophan could not be isolated from the urines by the method described by these authors would seem to support the view that orally administered acetyl-L- or acetyl-DL-tryptophan may be utilized by man. The analyses of these urines for free phenols by the method of Marenzi (4) and N^1-methylnicotinamide by the procedure of Huff and Perlzweig (5) indicate that neither isomer of acetyltryptophan is converted to these substances. This is of interest in view of the ready metabolic conversion of L-tryptophan to pyridine derivatives reported by others (7).

TABLE I

Urinary Output of Some Aromatic Metabolites for 24 Hour Period Following Oral Administration of 0.01 Mole of Acetyl-L- or Acetyl-DL-tryptophan

Subject A, male, 70 kilos.

Substance fed	Acetyltryptophan*		Free phenols	N^1-Methylnicotinamide
	Phenol reaction	Aldehyde reaction		
	mg.	mg.	mg.	mg.
None	1710	100	545	10.3
Acetyl-L-tryptophan	1722	105	450	9.1
Acetyl-DL-tryptophan	1790	110	545	8.2

Acetyltryptophan was determined by the method of Albanese, Frankston, and Irby (1), free phenols by the procedures of Marenzi (4) and Schmidt (4), and N^1-methylnicotinamide according to Huff and Perlzweig (5).

* It is to be noted, as Luck and associates have pointed out, that the reactions which they employed are not specific for acetyltryptophan. Moreover, the high phenol reaction value found is not as excessive as it appears when the number of urinary substances which react positively with the Folin reagent are considered (6).

Nitrogen Retention and Growth Experiments—In a previous report (8) we have shown that the infant can be maintained in normal growth and nitrogen retention on a complete diet which supplies a minimum of 40 mg. of L-tryptophan per kilo of body weight per day. With this information on hand, we felt that the availability of the derivatives for the support of these body functions at normal levels could be determined by supplementing the tryptophan-deficient diet with equivalent amounts of acetyl-L- or acetyl-DL-tryptophan. The biological value of the D component of racemic tryptophan was also tested by this technique.

The observations reported here were made on three normal healthy male infants who were given the synthetic diets in five feedings daily at the rate of about 100 calories per kilo of body weight. They were also given 50 mg. of ascorbic acid and 15 drops of oleum percomorphum daily. The diet periods were of 4 days duration and were consecutive. The subjects were immobilized by the use of abdominal restraints and 24 hour specimens were collected by means of adapters in bottles containing 10 cc. of 15 per cent (by volume) HCl and 1 cc. of 10 per cent alcoholic thymol. The feces were collected in 19 cm. porcelain evaporating dishes held in place by especially constructed mattresses and accumulated under refrigeration for each period in jars containing 200 cc. of 70 per cent alcohol. The infants were weighed daily during the course of the experiment.

The composition of the diets employed for the assay is shown in Table II. These were made to contain approximately 100 calories per 100 gm. and have the following percentile caloric distribution: protein 14, fats 36, carbohydrate 50. The protein moiety of the tryptophan deficient diet was prepared by sulfuric acid hydrolysis of casein as previously described by us (9). In order to improve the cystine-poor characteristic of this preparation, the final product was reinforced with 1 per cent L-cystine of the protein content estimated as N × 6.25. Owing to uncertainties regarding the complete human requirements of B complex vitamins, brewers' yeast was employed instead of a mixture of the synthetically available vitamins. The amount of L-tryptophan derived from this source appears to be approximately 6 mg. per gm. (10). Thus the quantity of L-tryptophan provided by the diets per kilo of body weight can be roughly estimated (Table II). The final nitrogen content of each batch of diet was determined by micro-Kjeldahl analysis.

The nitrogen retention data were obtained from the results of micro-Kjeldahl analyses of the daily 24 hour urine collections, period pools of the feces, and daily N intake as computed from the consumption record and nitrogen content of the diet.

The data thus obtained are collected in Table III and show that only Diet B failed to maintain nitrogen retention and body weight gain at the

TABLE II

Composition of Diets

Constituents	Diet A	Diet B	Diet C	Diet D	Diet E
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Acid-hydrolyzed casein*	3.4	3.4	3.4	3.4	3.4
L-Tryptophan	0.053	0	0	0	0.034
DL-Tryptophan	0	0.040	0	0	0
Acetyl-L-tryptophan	0	0	0.048	0	0
Acetyl-DL-tryptophan	0	0	0	0.048	0
L-Cystine	0.035	0.035	0.035	0.035	0.035
Brewers' yeast	1.0	1.0	1.0	1.0	1.0
Olive oil	4.0	4.0	4.0	4.0	4.0
Dextri-maltose No. 2†	9.6	9.6	9.6	9.6	9.6
Arrowroot starch	2.3	2.3	2.3	2.3	2.3
Salt mixture‡	1.6	1.6	1.6	1.6	1.6
Water	78.0	78.0	78.0	78.0	78.0
Total	100.0	100.0	100.0	100.0	100.0
Estimated L-tryptophan content, *mg.*	59.0	26.0	26.0	26.0	40.0

* N × 6.25 = gm. of protein.

† Kindly supplied by Mead Johnson and Company.

‡ The salt mixture employed had the following composition (measured in gm.): $FeSO_4$ 0.9, NaCl 6, calcium gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, MgO 0.1.

TABLE III

Effect of Optical Isomers of Tryptophan and Acetyltryptophan on Nitrogen Retention and Body Weight of Infant

The supplements were administered per kilo of body weight. All results given as daily averages.

Initial weight and age of subject		Diet A	Diet B	Diet C	Diet D	Diet E
		53 mg. L-tryptophan	40 mg. DL-tryptophan	48 mg. acetyl-DL-tryptophan	48 mg. acetyl-L-tryptophan	34 mg. L-tryptophan
R. M., ♂, 7 mos., 5.409 kilos	N intake, *gm.*	2.90	2.90	2.94	2.96	2.90
	" retention, *mg. per kg.*	160	96	132	136	120
	Weight change, *gm.*	+18	−9	+16	+19	+14
D. C., ♂, 13 mos., 7.347 kilos	N intake, *gm.*	3.14	3.00	3.12	3.10	3.14
	" retention, *mg. per kg.*	146	50	164	127	140
	Weight change, *gm.*	+16	+4	+18	+20	+20
H. G., ♂, 10 mos., 7.418 kilos	N intake, *gm.*	3.54	3.50	3.48	3.46	3.54
	" retention, *mg. per kg.*	130	66	120	132	148
	Weight change, *gm.*	+15	+3	+12	+18	+23

levels attained on Diet A. Since the growing organism is normally in a state of high nitrogen retention, a fall from the level characteristic of the individual must be given the same interpretation as the inducement of a negative nitrogen balance in the adult; namely, that, unlike acetyl-L- and acetyl-DL-tryptophan, the D component of DL-tryptophan is not utilized by man. This inference is further corroborated by the variations in body weight changes induced by the diets. These findings would seem to strengthen the deductions previously made from measurements of urinary products regarding the metabolic fate in man of D-tryptophan and both isomers of acetyltryptophan.

Comments

It is evident from our previous and present observations and those of Luck and coworkers that the amount of acetyltryptophan utilizable by man is dependent in a large measure on the mode of administration. The poor utilization of intravenously administered acetyltryptophan may be ascribed to two principal causes, (*a*) the parenteral route by passes some enzyme system present in the intestinal walls which can convert both optical varieties of the tryptophan derivative to utilizable indole substances, or (*b*) the rapid excretion of the injected compounds from the circulatory system into the urine precludes the action of any converting mechanism which the blood may contain. The latter possibility would seem more plausible in view of the fact that Luck and his collaborators were able to isolate unresolved racemic acetyltryptophan from the urine of their subjects.

The fact that the oral administration of acetyltryptophan, unlike L-tryptophan (6) does not cause an increase in the output of N^1-methylnicotinamide is of interest and is being further investigated.

SUMMARY

The earlier suggestion that orally administered acetyltryptophan is utilized by man has been strengthened by new measurements on the aromatic components of the urine. Additional support for the view is derived from body weight and N retention data obtained with infants maintained on diets in which the acetyltryptophan isomers constituted the major source of tryptophan. The D component of DL-tryptophan does not seem available for these functions.

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CREATINE AND CREATININE METABOLISM IN THE NORMAL MALE ADULT STUDIED WITH THE AID OF ISOTOPIC NITROGEN

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Experiments designed to demonstrate balance between the amount of creatine ingested and the amount retained or excreted as creatine and creatinine have so far failed in their purpose. The results of such studies have led to the opinion either that there is an end-product of creatine other than creatinine or that the synthesis of endogenous creatine is retarded by exogenous creatine (1).

We have reinvestigated the question of creatine balance by means of isotope methods. By labeling the tissue creatine of a human subject with N^{15} it has been possible to observe the changes in the amount of creatine deposited in the body after the ingestion of non-isotopic creatine. At the same time, the relationship between the amount of creatine present in the tissues at any time and the amount excreted as creatinine has been studied. Finally, the rate of turnover of body creatine was measured before and after the administration of exogenous creatine.

Bloch, Schoenheimer, and Rittenberg (2) have shown that the creatinine excreted in the urine of rats fed isotopic creatine while subsisting on a creatine-free diet must have been derived from body creatine, since the concentrations of N^{15} in the creatinine and the muscle creatine were identical. Experimental confirmation through the use of deuteriocreatine has been provided by du Vigneaud and his collaborators (3, 4). In view of these observations we have used the isotope concentration of the creatinine isolated from the urine of the subject to calculate changes of body creatine.

EXPERIMENTAL

Isotopic Creatine—Isotopic creatine was synthesized from glycine containing 31.5 atom per cent excess N^{15} by the methods described by Bloch *et al.* (5);[1] N^{15} found, 10.3 atom per cent excess; N^{15} calculated, 10.5 atom per cent excess; H_2O of hydration found, 12.01 per cent; H_2O of hydration calculated, 12.06 per cent.[2] The non-isotopic creatine used was recrystallized C.P. creatine obtained from commercial sources; H_2O of hydration found, 12.10 per cent.

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† James Hudson Brown Memorial Research Fellow.

[1] We wish to thank the American Cyanamid Company, Stamford, Connecticut, for their generous supply of cyanamid.

Isolation Procedure—Creatinine was isolated from urine according to the method of Benedict (6). The recrystallized creatinine zinc chloride was treated overnight with 10 per cent barium hydroxide solution under reflux. After removal of barium as the sulfate, sarcosine was isolated as the toluene sulfonyl derivative. Two recrystallizations from water assured purity of the product as determined by the melting point. The isolation of toluene sulfonyl sarcosine avoided dilution of isotopic nitrogen by non-isotopic amidine nitrogen.

Analyses—Urine analyses for creatine and creatinine were performed by the method of Peters (7). Urine was collected daily throughout the entire experimental period. Usually 4 day pools were analyzed for creatine and creatinine. On the 5th day separate aliquots were taken for these analyses and for creatinine isolation. 1 ml. of 10 per cent thymol in ethanol was added to the bottle used for each day's collection.

Analyses for guanidoacetic acid were carried out according to the method described by Hoberman (8).

We are greatly indebted to Dr. D. Rittenberg of the Department of Biochemistry, Columbia University, for his kindness in permitting one of us (H. D. H.) to carry out N^{15} analyses in his laboratory.

Diet—10 days before the ingestion of isotopic creatine, the subject (E. A. H. S.) was placed on a diet free of meat or meat products. Milk consumption was limited to 250 ml. per day. On an intake of 90 gm. or more of protein and of 2500 calories or more per day, the subject was able to maintain his weight (63 kilos) to within 1 kilo.

Creatine Ingestion—1.295 gm. of N^{15} creatine (20 mg. per kilo) were dissolved in 500 ml. of water and taken in equal doses every 30 minutes for 10 hours. Urine collection, begun at the time of ingestion, was terminated in 24 hours. No detectable creatinuria was found. Each day thereafter for 38 days urine was collected and treated as described under "Analyses." On the 39th day the N^{15} content of the body creatine was raised by the ingestion of 1.16 gm. of guanidoacetic acid containing 30.5 atom per cent excess N^{15} in the glycine moiety. During the 10 days which followed urine was collected as usual, and treated in the manner described under "Analyses." On the 11th day following the ingestion of N^{15} guanidoacetic acid, the ingestion of non-isotopic creatine was begun.

27.33 gm. of creatine hydrate (24.00 gm. of creatine) were dissolved in 2 liters of water. On each day for 5 consecutive days 400 ml. of the solution described above (4.8 gm. of creatine, 75 mg. per kilo) were consumed. The creatine was taken in equal portions at 30 minute intervals from 7 A.M. until 11 P.M. The urine of the 3rd, 4th, and 5th days contained respectively 0.15, 0.52, and 0.81 gm. of creatine, indicating that 94 per cent of the dose had been retained. Creatine did not appear in the urine after the 5th day of this experiment. Analyses for creatine and creatinine and analyses of the urinary creatinine for isotope were carried out as described under "Analyses" for a period of 28 days.

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[2] Calculated for 10.3 atom per cent excess N^{15}.

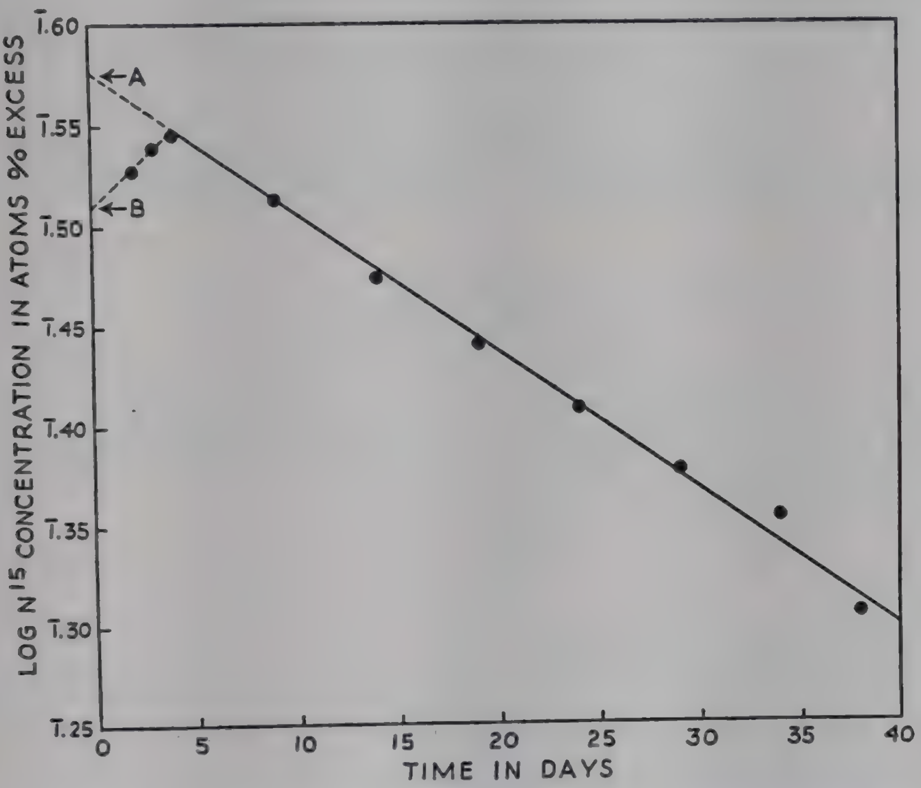

FIG. 1. Rate of turnover of body creatine on a creatine-creatinine-free diet. *A* = the extrapolated value of the log of the N^{15} concentration for mixing at zero time; *B* = the extrapolated value of the log of the N^{15} concentration at the end of the day of creatine ingestion.

Results

Creatine Turnover on Creatine-Free Diet—In Fig. 1 is a semilogarithmic graph of the data obtained after the ingestion of the isotopic creatine. That the slope of the straight line obtained is equal to the rate of turnover of body creatine may be proved as follows.

Consider the addition of X gm. of isotopic creatine to G gm. of non-isotopic body creatine. Provided that X is much smaller than G,

$$(1) \qquad \frac{C}{100} = \frac{X}{G}$$

where C is the isotope concentration in atom per cent excess of the mixture. Provided that the rate of uptake of newly synthesized creatine is equal to the rate of loss of creatine as creatinine, G may be treated as a constant, and

$$(2) \qquad \frac{1}{100}\frac{dC}{dt} = \frac{1}{G}\frac{dX}{dt}$$

After ingestion, absorption, and mixing of the isotopic creatine are complete,

$$(3) \qquad \frac{dX}{dt} = -\frac{e}{C}100$$

That is, the rate of loss of isotopic creatine is equal to the number of gm. per day of creatine, e, excreted as creatinine, multiplied by the fraction present as the isotopic compound. Then

$$(4) \qquad \frac{dC}{dt} = -\frac{e}{G}C$$

and integrated from $t = 0$, and $C = C_0$

$$(5) \qquad ln\ \frac{C_0}{C} = \frac{c}{G}t$$

e/G represents the fraction of creatine synthesized and excreted per day. This is the slope, k, of the curve of Fig. 1, and is computed to be 0.0164 per day. Since the average daily output of creatinine during the experimental period was 1.89 gm. of creatine (1.63 gm. of creatinine), the total creatine content, G, of the subject is $1.89/0.0164 = 115$ gm. A check on this figure is obtained by calculating the total creatine content from the isotope concentration if mixing had occurred at zero time. This concentration is obtained by extrapolation of the curve of Fig. 1 to zero time. Point A is equivalent to an isotope concentration of 0.376 atom per cent excess. As shown below, 97 per cent of the isotopic creatine was retained, so that $0.97 \times 1.295 = 1.26$ gm. of creatine were available for mixing with the creatine of the body tissues. Then $G = (31.5 \times 1.26/(0.376)) - 1.26 = 105$ gm. in good agreement with the quantity calculated above.

It will be noted that creatine is turned over somewhat more slowly in the human than in the rat. The time of 50 per cent turnover calculated from

$t_{\frac{1}{2}} = 2.3/k \log 2$ is 42 days, in comparison with 29 (2) and 36 days (4) reported for the rat.

In order to determine to what extent degradation of the administered creatine had occurred, ammonia and urea were isolated from the urine collected during the day of ingestion. The isotope concentrations of these compounds and of the stool nitrogen of the first 2 experimental days are tabulated below (Table I).

Ammonia was isolated by adsorption on and distillation from permutit. Urea was isolated as xanthydryl urea.

From these data it appears that 3 per cent of the creatine given can be accounted for in nitrogenous substances other than creatinine.

Creatine to Creatinine Transformation—Particularly because the isotope concentration of the urine creatinine has been used to calculate changes in the amount of body creatine, it is necessary to show that there is little or no conversion of creatine to creatinine until the former has become mixed with the creatine of the body. That this is the case under the conditions of the experiment described is indicated by the following analysis. By extrapolation of the curve of Fig. 1 to Point B, the N^{15} concentration of the creatinine excreted at the end of the first experimental day was 0.324 atom per cent excess. The isotope concentration of the creatinine excreted during this day was the average of values between 0 and 0.324 atom per cent excess. Since the isotopic creatine was ingested at a constant rate over a 10 hour period, it may be assumed that during this period the isotope concentration in the creatinine averaged approximately one-half of 0.324 atom per cent excess, or 0.162 atom per cent excess. For the remainder of the day the isotope concentration of the creatinine averaged approximately 0.324 atom per cent excess. The average concentration for the entire day was, therefore, $0.162 \times 10/24 + 0.324 \times 14/24$, or 0.255 atom per cent

TABLE I

Concentration of N^{15} in Urinary Constituents and in Stool Nitrogen after Ingestion of Isotopic Creatine

Substance	N	N^{15} concentration, atom per cent excess	N^{15} creatine
	mg.		mg.
Ammonia	105	0.013	0.42
Urea		0	*
Stool 1	1320	0.042	17.0
" 2	2000	0.028	17.1

* Had as little as 15 mg. of the ingested creatine been degraded to urea, a measurable excess of N^{15} would have been found in the presence of 10 gm. of urea nitrogen

excess. The observed concentration, 0.226 atom per cent excess, is, therefore, too low to include creatinine formed by conversion of the administered creatine before mixing had been attained. Moreover, if all of the isotope in the creatinine excreted during the first experimental day had been derived from the isotopic creatine before dilution occurred, only 1 per cent of the amount given would have been required to produce the observed N^{15} concentration.

Bloch and Schoenheimer (5), treating their data in a similar manner, found that the isotope concentration of the creatinine isolated from the urine of rats fed isotopic creatine was somewhat higher than the calculated value. Approximately 2 per cent of the ingested creatine may have been converted to creatinine before dilution with all of the body creatine had occurred.

Effect of Exogenous Creatine on Turnover—Once N^{15} creatine has been deposited in the tissues of the body and the rate of replacement of body creatine has been determined in the manner described, the effect of exogenous creatine on the turnover may be studied by measuring the dilution of isotope resulting from the ingestion of the non-isotopic substance.

The changes in the N^{15} concentration of the creatinine excreted during and after the ingestion of 24 gm. of non-isotopic creatine are shown in Fig. 2. N^{15} became rapidly diluted during, and for 2 days after, the ingestion period. A marked decrease in slope followed, with return to the original rate of dilution approximately 17 days after the start of the experiment. The significance of these changes becomes clearer when the N^{15} concentrations and corresponding times are used to calculate the total body creatine by means of an equation developed as follows:

The complete differential of equation (1) with respect to time is

$$\frac{1}{100}\frac{dC}{dt} = \frac{G\frac{dX}{dt} - X\frac{dG}{dt}}{G^2} \tag{6}$$

By substituting the value of X from equation (1) and of dX/dt from equation (3), and by simplifying,

$$\frac{d\ln C}{dt} = \frac{e}{G} + \frac{d\ln G}{dt} \tag{7}$$

If it is now assumed that $e/G = k$, or, in other terms, that the amount of creatine excreted as creatinine is proportional to the total amount of creatine in the body, equation (7) may be integrated between $t = 0$, and t, C_0, and C, and G_0 and G to yield

$$G = \frac{C_0 G_0}{C} e^{-kt} \tag{8}$$

By letting $C_0 = 0.356$ atom per cent excess,[3] $G^0 = 115$ gm., and $k = 0.0164$ per day, the values of G have been calculated for the observed values of C and t (Table II, Column A).

These are compared with the values of G which would have resulted if all of the ingested creatine had been deposited in the tissues of the body (less the 1.5 gm. excreted in the urine), and if the synthesis and uptake of endogenous creatine had been independent of the amount of exogenous creatine (Table II, Column B). Under these circumstances the changes in body creatine are described by the following differential equation:

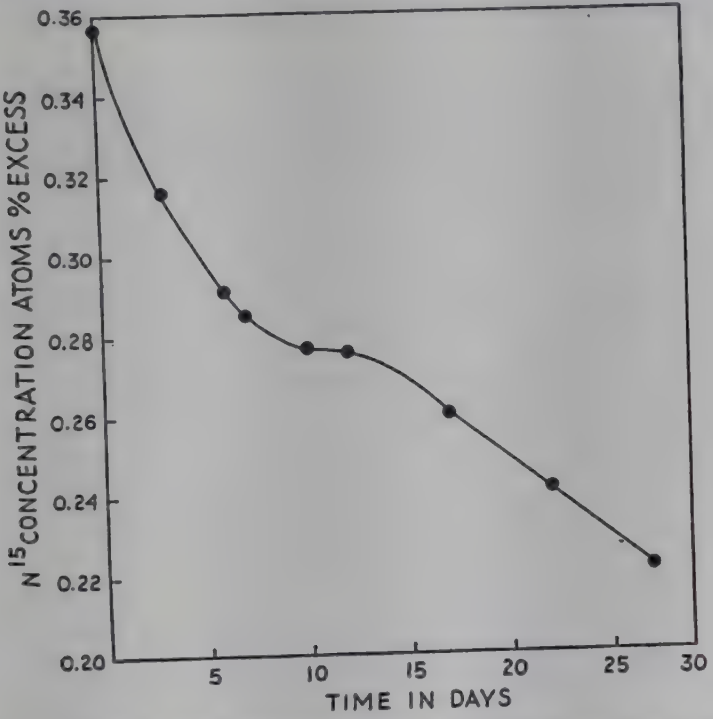

FIG. 2. Changes in the N^{15} concentration of urinary creatinine during and after the ingestion of 24 gm. of non-isotopic creatine.

$$\frac{dG}{dt} = a + b - kG \tag{9}$$

where a = gm. of exogenous creatine deposited per day, b = gm. of endogenous creatine deposited per day, k = fraction of total creatine excreted as creatinine per day, and G = gm. of body creatine. The integrated expression from $t = 0$, and $G = C_0$ is

$$G = \frac{a + b}{k}\left(1 - e^{-kt}\right) + C_0 \cdot e^{-kt} \tag{10}$$

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[3] This is the N^{15} content of the tissue creatine on the 10th day after ingestion of N^{15} guanidoacetic acid. It should also be mentioned that, during the 10 days following the ingestion of N^{15} guanidoacetic acid, the rate of turnover of body creatine was identical with that found during the antecedent 38 day period.

With $k = 0.0164$ per day, $G_0 = 115$ gm., $a = 4.8$ gm. per day (less the amounts lost in the urine on the 3rd, 4th, and 5th days), and $b = 1.89$ gm. per day, G has been calculated from $t = 0$ to $t = 5$. From $t = 6$ to $t = 27$, G has been calculated for $a = 0$, with $G_0 = G$ for $t = 5$.

From the results in Table II it is evident that the uptake of exogenous creatine fell short of the calculated amount. Whereas the application of equation (10) leads to an expected uptake of 22 gm. of the 24 gm. administered, the observed increase was only 13 gm., 59 per cent of the calculated maximum.

A second noteworthy difference between the data listed in Columns A and B, Table II, is the rate of decrease of body creatine. Whereas approximately 10 days were required for the tissue creatine to fall from 128 to 119 gm., 73 days would have been required for the same loss to have occurred according to the conditions necessary for the application of equation (10). It should be mentioned that the apparent decrease in body creatine occurred with no associated increase in the amount of creatinine excreted.

TABLE II

Changes in Body Creatine, during and after Ingestion of 24 Gm. of Non-Isotopic Creatine

Time	N^{15} concentration, atom per cent excess	Total creatine, observed	Total creatine, calculated
		(A)	(B)
days		*gm.*	*gm.*
0	0.356	115	115
3	0.316	123	129
5			137
6	0.291	128	
7	0.285	128	
10	0.277	126	136
12	0.276	122	
17	0.260	119	133
22	0.241	118	132
27	0.221	119	130

It should also be pointed out that not only did the deposition of creatine continue to appear, albeit at a decreasing rate, in the presence of an increasing creatinuria but also after the last dose of exogenous creatine had been administered.

Creatinine Excretion—Graphical integration of the data in Table II, Column A, permits calculation of the average creatinine excretion according to

$$\bar{e} = k\bar{G} \tag{11}$$

where $\bar{e}$ is the average creatinine excretion (expressed as gm. of creatine) and $\bar{G}$ is the average body creatine content, the integration being performed over the 27 day experimental period. By this method $\bar{e}$ was calculated to be 2.00 gm. per day.

The average creatinine excretion which would be expected to result from the increase in body creatine calculated by means of equation (10) can be directly computed, since

$$\bar{G} = \frac{1}{t} \int_{t=0}^{t} G \, dt \tag{12}$$

Solving equation (12) and substituting in equation (11), we find the average creatinine output (expressed as gm. of creatine) to be 2.20 gm. per day. This is in contrast with the observed average creatinine excretion, which was equivalent to 2.05 gm. of creatine, in good agreement with the amount calculated by graphical integration of the isotope dilution data.

Creatine to Creatinine Transformation—Although the direct conversion of creatine to creatinine was shown to be improbable for the experiment in which a relatively small dose of isotopic creatine was given, it has not yet been proved that this holds true under the conditions of the experiments just described. The possibility of direct conversion should increase with the concentration of creatine in the serum, and therefore during creatinuria. Actually, although the excretion of creatine during the 4th and 5th days of creatine ingestion was almost 10 times as great as its excretion during the first 3 days, the rate of dilution of the isotope was greater during the first 3 than it was for the second 3 days.

Effect of Creatine Ingestion on Excretion of Guanidoacetic Acid—The differences encountered between the amounts of creatine calculated from isotope dilution data on the one hand and from equation (10) on the other and the discrepancy between the observed and calculated excretion of creatinine indicate that the assumptions upon which the validity of equation (10) depends are questionable. In order to test the possibility that exogenous creatine influences the rate of synthesis of creatine, the excretion of guanidoacetic acid in urine was measured before and after the ingestion of creatine. It was reasoned that any retardation of the synthesis of creatine might be reflected in an increase of the excretion of guanidoacetic acid, the immediate precursor of creatine (9).

That the amount of guanidoacetic acid excreted in the urine increases after creatine ingestion is seen in Fig. 3. Oral tolerance to guanidoacetic acid is lowered significantly when the guanidoacetic acid is ingested with creatine. This is illustrated in Fig. 4, which shows the amount of guanidoacetic acid excreted in 24 hours when 2.5 gm. of creatine were taken alone (*A*), when 1.5 gm. of guanidoacetic acid were taken alone (*B*), and

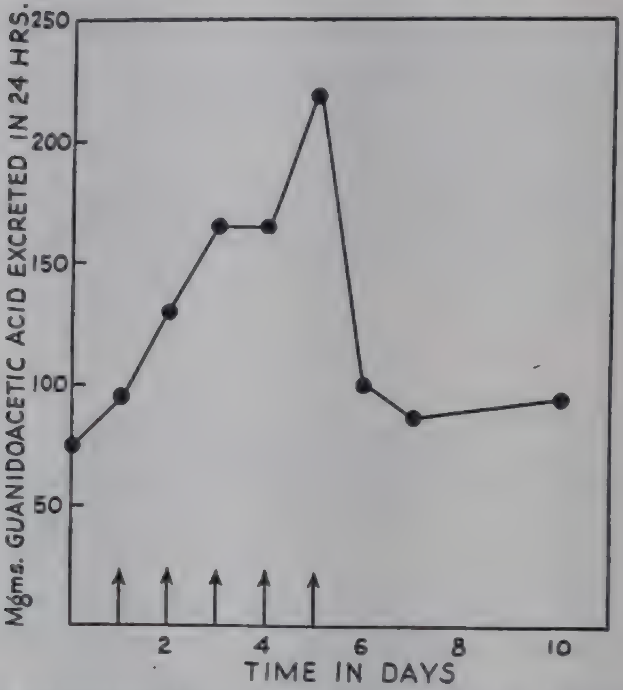

FIG. 3. Excretion of guanidoacetic acid during creatine ingestion. The arrows denote the dose of 4.8 gm. of creatine per day.

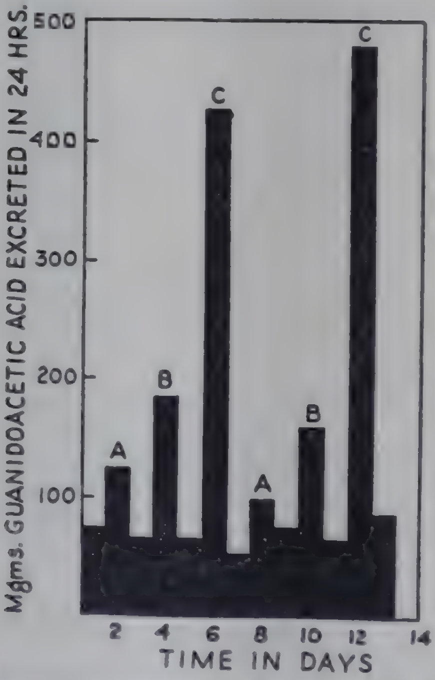

FIG. 4. Excretion of guanidoacetic acid when (*A*) 2.5 gm. of creatine ingested alone; (*B*) 1.5 gm of guanidoacetic acid ingested alone; (*C*) 2.5 gm. of creatine and 1.5 gm. of guanidoacetic acid ingested together.

when guanidoacetic acid and creatine were ingested together (*C*). The simultaneous ingestion of the two compounds increased the guanidoacetic acid content of the urine more than 100 per cent over the control value,

DISCUSSION

Creatine Balance—The present study shows that exogenous creatine may be completely accounted for neither in an increase of body creatine nor by extra creatinine excreted. Creatine apparently disappeared not only during the period of ingestion, but also during the subsequent 10 days. Any single theory proposing to account for the facts would have to be equally applicable to both events. For example, any argument based upon incompleteness of absorption of the ingested creatine to account for the loss incurred during the ingestion period could not apply to events which transpired during the post-ingestion period. Similarly it would be difficult to account for the observations described on the theory that creatine was destroyed, since the destructive process would be required to apply to substrate both before and after deposition. Tracer studies have shown that in the rat destruction of creatine is improbable. After daily doses of N^{15} creatine 50 per cent greater in amount than the normal daily turnover, no important metabolic end-product of creatine other than creatinine could be demonstrated (2). Among the conditions upon which the validity of equation (10) depends is the stipulation that the rate of synthesis of endogenous creatine is independent of the amount of exogenous creatine. The results of the experiments described are more compatible with the hypothesis that the synthesis of creatine is retarded by, or in the presence of, exogenous creatine. Such a process would yield the results observed here.

The increase in the excretion of guanidoacetic acid after creatine administration accounts at best for only a few per cent of the administered material. Unless another metabolic pathway exists for guanidoacetic acid, it would be difficult to accept the excretion of extra guanidoacetic acid as evidence for a theory of "backing up" of synthesis. Evidence is at hand, however, which shows that creatine deposited in the tissues of the body after N^{15} guanidoacetic acid is ingested accounts for only 70 per cent of the guanidoacetic acid given. Significant amounts of N^{15} were found in the urea and ammonia of the urine. After the intravenous injection of a similar amount of N^{15} guanidoacetic acid the concentration of N^{15} in the urinary ammonia and urea was equivalent to that found after ingestion of the compound.[4] It is therefore apparent that in man a significant amount of guanidoacetic acid may enter into reactions *in vivo* other than conversion to creatine.

The nature of the retardation process can only be suggested at this time. That the methylation of guanidoacetic acid is irreversible seems well established (2, 10). There is, however, a type of chemical reaction which could account for the retardation of creatine synthesis on another basis.

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[4] Unpublished observations.

The participation of creatine in a competitive reaction is such a possibility. It has recently been shown that the synthesis of creatine from guanidoacetic acid and methionine may require the presence of adenosine triphosphate (11). It is conceivable that exogenous creatine may act here as a competitive substrate for adenosine triphosphate, depriving the synthetic mechanism of a requisite high energy phosphate bond.

The presence of creatine in especially high concentration may retard the methylation of guanidoacetic acid to such an extent that extra guanidoacetic acid appears in the urine. When the degree of inhibition is somewhat less, the accumulation of guanidoacetic acid may be prevented by a reversal or retardation of the processes by which this compound is formed. These hypotheses are now being investigated in this laboratory.

Transformation of Creatine to Creatinine—The non-enzymatic dephosphorylation of creatine phosphate to creatinine has been demonstrated to take place in aqueous solution at a rate sufficiently fast to account for the amount of creatinine formed and excreted per day (12). The results of the experiments described in this paper are consistent with such a theory of creatinine formation, since they indicate that the amount of creatinine excreted per day is directly proportional to the amount of creatine present in the body. The amount of creatine, existing as phosphate, which must be present at any time, calculated from Borsook's data together with ours, is approximately 35 per cent of the total. Since this is significantly lower than the actual amount present, it would seem likely that Borsook's value for the rate constant of the reaction creatine phosphate → creatinine is lower *in vivo* than *in vitro*. It is likely that his assumption of irreversibility of the reaction creatine phosphate → creatine + inorganic phosphate does not apply to the system *in vivo*, since the splitting of phosphocreatine to creatine is reversible in muscle (13). It is also possible, however, that another process, as yet undescribed, determines the rate of creatinine formation.

Rate of Creatine Uptake—The spontaneous conversion of creatine to creatinine, had it occurred before mixing with the creatine of the body and had it taken place at the rate reported for the reaction *in vitro* (12), could completely account for the N^{15} concentration of the creatinine isolated from the urine collected during the day of ingestion of isotopic creatine. The fact that this was not the case is convincing evidence for a rapid rate of turnover of phosphocreatine in the body. The rapidity of this exchange has been repeatedly demonstrated with radiophosphorus as a tracer (14–16). That the apparent rate of uptake of creatine is determined by the exchange of phosphate within cells or during the passage of creatine across the cell membrane is therefore unlikely. The fact that there was continued uptake of creatine in the presence of increasing creatinuria as well as after the cessation of creatine feeding is evidence, however, of the existence of a rate-setting process involved in the deposition of exogenous creatine. The isotope concentration of creatine isolated from the internal organs of rats sacrificed shortly after being fed N^{15} creatine was approximately 15 per cent greater than that of the muscles (5). This observation is consistent with the theory that the internal organs act as a reservoir for exogenous creatine. The slow process observed might therefore be related to transportation of creatine between organs. If the conversion of creatine to creatinine had taken place at the same rate in the internal organs as in muscle, the creatinine excreted would have the same isotope composition as if interorgan mixing had occurred. Since this was not the case, either the rate of conversion of creatine to creatinine in the internal organs was slower than the rate in muscle, or the transport of creatine from internal organs to muscle is rate-determining. Since the minimal rate of conversion of creatine to creatinine is in all probability the non-enzymatic dehydration of creatine, which, it has been pointed out, was not detectable in our experiments, it is most probable that the process which determines the rate of mixing is related to the transportation of creatine between organs.

That there are at least two reservoirs for creatine, one expansible (muscle) and the other of relatively limited capacity (liver, kidneys) is likely in view of the fact that the deposition of creatine continued during increasing creatinuria. Creatinuria, it would follow, occurs when the rate of filling of the reservoir of limited capacity exceeds the rate of transport of creatine to the more expansible one. That the deposition of creatine continued after the cessation of creatine ingestion might well be due to the transport of creatine from the filled reservoir (liver, kidneys) to the unfilled one (muscle). The process or processes involved in interorgan mixing cannot be identified at this time. That the observed rate of mixing is slower than the speed of circulatory transmission can be calculated. It is suggested that the step which determines the rate of interorgan mixing may be concerned with the reaction in muscle, creatine (extracellular) $\rightleftharpoons$ creatine (intracellular). It is also suggested that this step may depend upon the speed of uptake or the availability of inorganic phosphate.

SUMMARY

By labeling the tissue creatine of a human subject with N^{15} the changes in the amount of creatine deposited in the body have been observed before and after the ingestion of a large dose of the non-isotopic compound. It was found that the rate of turnover of endogenous creatine on a diet as free of creatine and creatinine as possible is 1.64 per cent per day.

Balance could not be demonstrated between the amount of creatine

retained and the amount actually deposited in the depot, or excreted as extra creatinine. To explain these observations it is suggested that the synthesis of endogenous creatine is retarded in the presence of exogenous creatine.

The amount of creatinine excreted daily was shown to be directly proportional to the amount of creatine in the body.

The uptake of creatine by muscle takes place at a measurable rate. The processes which may be involved in determining this rate are discussed.

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NUCLEIC ACIDS AS GROWTH FACTORS IN DROSOPHILA

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Investigators have been trying for many years to obtain a chemically defined medium which will support the growth of insects. There were two main reasons for this search. Some men were interested in it as a problem in comparative nutrition, and many interesting differences between vertebrate and insect requirements have come to light (*cf.* Trager's review (1)). Geneticists were interested in obtaining a constant, defined medium for exact studies of gene expression, since the expression of many of the genes of *Drosophila*, *Ephestia*, and other insects vary with the nutritional state of the animal. The work of Beadle and Tatum (2) on the biochemical mutants of *Neurospora* has given added impetus to the search, since a chemically defined medium would permit an investigation of similar mutants in *Drosophila*. The requirements of insects for various substances have been worked out in several different studies: salts (3), cholesterol (4), thiamine and riboflavin (5), and pyridoxine, pantothenic acid, and niacin (6). Finally, Schultz *et al.* (7) announced a chemically defined medium which would allow the growth of *Drosophila* in the absence of microorganisms. Pupation on this medium occurs in 8 to 9 days at 25° instead of in 4 to 5 days on a yeast medium. The water-insoluble fraction of yeast contains some as yet unidentified substance necessary for rapid development. Ribonucleic acid was one of the components important for growth.

The present paper reports the effects of substituting various nucleotides and purine or pyrimidine bases for the ribonucleic acid in a formula essentially similar to Schultz'.

Methods

The medium was prepared in two parts (Table I). Solution A, containing salts, agar, amino acids, cholesterol, etc., was made up in 437 ml. of water and sterilized by autoclaving at 15 pounds pressure for 20 minutes. Solution B, containing the vitamins, was made up in 63 ml. of water and sterilized by filtration through a medium sintered glass filter. These solutions were stored in the cold. In making up the culture tubes, 3.5 ml. of Solution A and 0.5 ml. of Solution B were transferred by sterile procedures to each tube, and the two were mixed together thoroughly and then allowed to cool on a slant.

Eggs were collected from half pint culture bottles within a 2 hour period, washed in insect-Ringer's solution to remove most of the adhering yeast, sterilized in 70 per cent ethanol (8), and transferred by sterile techniques, with a platinum loop and flaming, to the tubes containing the test medium. This technique resulted in sterile cultures in 95 to 97 per cent of the tubes set up. Results on the non-sterile tubes were omitted from the tables, since they were not comparable to the sterile ones. Ten to twenty eggs were placed in each tube containing 4 ml. of food. Two to five tubes of each medium for each stock were used. The tubes containing the developing flies were kept at 25° and examined every day.

TABLE I

Chemically Defined Medium for Drosophila

Solution A

Agar	7.5 gm.	KH_2PO_4	0.303 gm.
Casein hydrolysate	10.0 "	K_2HPO_4	0.303 "
L-Cystine	0.2 "	$CaCl_2$	0.00645 "
L-Tryptophan	0.1 "	$FeSO_4 \cdot 7H_2O$	0.00645 "
Cholesterol	0.05 "	$MgSO_4 \cdot 7H_2O$	0.123 "
Lecithin	0.05 "	$MnSO_4 \cdot 4H_2O$	0.00645 "
Ribonucleic acid	0.5 "	NaCl	0.00645 "
Sucrose	25.0 "	H_2O	437 ml.

Solution B

Biotin	7.5 γ	Ca pantothenate	3 mg.
Choline	10.0 mg.	Folic acid	3 "
p-Aminobenzoic acid	1.0 "	Pyridoxine HCl	1.5 "
Inositol	21.0 "	Riboflavin	1.2 "
Niacinamide	5.0 "	Thiamine HCl	0.75 "
		H_2O	63 ml.

Results

Effects of Substituting Nucleotides for Ribonucleic Acid (RNA)—0.5 gm. of RNA was hydrolyzed by incubation with 5 ml. of 1 N NaOH at 38° for 24 hours (9). It was then placed at 5° for 12 hours. The solution was then neutralized with 1 N HCl (with phenol red as indicator) and evaporated to dryness *in vacuo* at room temperature overnight. The light tan powder obtained was added to a batch of Solution A in place of ribonucleic acid, sterilized by autoclaving, and tubes containing 3.5 ml. of this solution and 0.5 ml. of Solution B were prepared.

The resulting growth of *Drosophila* on this medium compared with growth on the normal medium containing RNA, measured by the length of time required for pupation and hatching, is given in Table II. The figures are the average number of days from the time the eggs were placed on the medium until pupation occurred and until the adults hatched. The figures in parentheses are the ranges for these figures. In general, the flies grow as well on the nucleotide medium as on the RNA medium. Stocks of Bar and Double Bar (eyes) grow a little better on RNA, and stocks of four-jointed (legs) and tetraltera (wings) grow a little better on nucleotides. All of the mutants develop a little more slowly than wild type on these media, just as they do under normal culture conditions with corn-meal, molasses, agar, and yeast. The tetraltera flies raised on RNA never hatched out but died as pupae. With all the stocks on all the media tried, there usually was a considerable mortality in both the larval and pupal stages; only about 70 per cent of the larvae pupated and about 40 per cent hatched.

Table II

Growth of Drosophila on Ribonucleic Acid and on Nucleotides from Hydrolyzed Ribonucleic Acid

Stock[*]	Medium with ribonucleic acid, 1 mg. per ml.		Medium with nucleotides, 1 mg per ml.	
	Egg-pupa	Egg-adult	Egg-pupa	Egg-adult
	days	*days*	*days*	*days*
Wild	10.3 (9–13)[†]	14.5 (13–16)	10.9 (10–12)	14.2 (14–15)
vg	13.2 (12–15)	17.3 (16–18)	13.1 (12–14)	17.0 (16–18)
B	10.5 (9–13)	14.9 (14–16)	11.7 (10–13)	15.8 (14–17)
BB	10.6 (10–12)	15.3 (15–17)	12.1 (11–13)	16.2 (15–17)
ds	13.2 (11–15)	16.3 (15–18)	13.2 (11–15)	16.4 (15–18)
fj	12.9 (11–15)	16.1 (15–18)	12.1 (11–13)	16.0 (15–17)
ss^a	11.5 (10–13)	15.4 (14–17)	11.8 (11–13)	16.1 (15–17)
tet	17.0 (16–18)		14.2 (12–16)	17.2 (16–18)

[*] *vg*, vestigial wings; *B*, Bar eyes; *BB*, Double Bar eyes; *ds*, dachsous (short) legs; *fj*, four-jointed (short) legs; ss^a, aristapedia (antennae replaced by legs); *tet*, tetraltera (wings replaced by halteres).

[†] The figures in parentheses represent the range.

Effects of Substituting Purines and Pyrimidines.—The purines and pyrimidines which were available were tested singly and in combinations as substitutes for RNA in the medium. In one set of experiments (Table III), sucrose was omitted from the medium and in another set (Table IV) sucrose was added in normal amount. It can be seen from Table III that the omission of sucrose results in a drastic reduction in the percentages of flies pupating and hatching. One Double Bar larva was able to pupate on a medium containing neither sucrose nor nucleic acid after 21 days of larval development, and it hatched on the 25th day. This fly was the only phenocopy observed in the course of the experiments: the eyes, instead of being much reduced in size and bar-shaped as *BB* eyes normally are, were larger, approaching wild type in size, and were oval in shape.

TABLE III

Growth of Drosophila on Media Containing Certain Purines and Pyrimidines but No Sucrose

Purine or pyrimidine	Concentration	Wild			Vestigial	Bar			Double Bar				
		Larvae	Pupated		Larvae*	Larvae	Pupated		Larvae	Pupated		Adult	
	mg. per ml.	*days*	*days*	*per cent*	*days*	*days*	*days*	*per cent*	*days*	*days*	*per cent*	*days*	*per cent*
None		6–21		0	2–12	2–14		0	8–38	21	4	25	4
Adenine	1	1– 3		0	1– 2	1– 2		0	1– 2		0		0
" Ribose	1 0.3	3– 5		0	1– 2	1– 2		0	1– 2		0		0
Adenine Guanine Uracil	0.2 0.2 0.2	3–18	9–11	58	2–14	3–15	15	5	2–15	11–15	10		0
Adenine Guanine Uracil Ribose	0.2 0.2 0.2 0.2	3–17		0	1–12	1–24	15	5	3–19		0		0
Adenine Guanine	0.4 0.4	1–16		0	2–13	1–15		0	2–15		0		0
Adenine Uracil	0.4 0.4	2–21	11	14	2–19	2–12		0	2–12	10	11		0
Guanine	0.8	13–25		0	2–18	2–18		0	1–20		0		0
Desoxyribonucleic acid	0.3	2–28		0	2–20	2–12		0	2–14		0		0

* None pupated.

On the sugarless media (Table III), the larvae of all four stocks tested died very quickly on media containing only adenine or adenine plus ribose. They lived for long periods of time but were unable to pupate on media containing desoxyribonucleic acid, guanine, or guanine plus adenine. A few were able to pupate, but none hatched, on media containing adenine plus uracil, adenine, guanine, and uracil, or adenine, guanine, uracil, and ribose. Some differences in the ability of different stocks to grow on these media were observed. Vestigial larvae were unable to pupate on the medium (adenine, guanine, and uracil) which permitted 58 per cent of the wild type larvae to pupate. With adenine alone, the larvae died very quickly.

TABLE IV

Growth of Drosophila on Media Containing Certain Purines and Pyrimidines Plus 5 Per Cent Sucrose

Purine or pyrimidine	Concentration	Wild					Vestigial					Double Bar				
		Larvae	Pupated		Hatched		Larvae	Pupated		Hatched		Larvae	Pupated		Hatched	
	mg. per ml.	*days*	*days*	*per cent*	*days*	*per cent*	*days*	*days*	*per cent*	*days*	*per cent*	*days*	*days*	*per cent*	*days*	*per cent*
None		2–26	13.1	39	17.3	25	3–16	12.2	55	15.7	44	2–36	10.3	74	14.2	64
Adenine	1	3–11	8.2	50	11.4	28	3–11	10.2	23	14.5	12	2–11	9.3	72	13.4	46
" Guanine Uracil	0.5 0.5 0.5	5–15	8.6	88	11.6	76	1–13	10.2	66	14.3	50	2–10	8.5	87	11.0	81
Guanine Uracil	0.5 0.5	5–20	16.5	92	18.9	54	2–31	17.5	21	22.0	4					
RNA Benzimidazole	1 1	1– 2		0		0	1–23		0		0	1– 2		0		0
Adenine Benzimidazole	1 1	1– 2		0		0	1–21		0		0	1– 2		0		0
"	1	1		0		0	1–15	14.8	20	19.0	5	1		0		0
"	0.1	5–21	15.1	69	18.0	54	10–18	13.1	90	17.4	55					

With guanine alone, the larvae lived up to 25 days but were small and never pupated. With adenine plus guanine, the larvae lived longer than on adenine alone and grew to almost normal size but could not pupate. With adenine plus uracil the larvae lived long, were of almost normal size, and a few wild type and Double Bar larvae pupated. With adenine, guanine, and uracil, the larvae grew to normal size and more than half of the wild type flies pupated in about the same time as they would have on a normal nucleic acid-sucrose medium.

When sugar is added to the medium (Table IV), the larvae of wild, vestigial, and Double Bar are able to pupate and hatch in the absence of RNA. When purines or pyrimidines are added, the larvae pupate in a shorter time and a greater percentage of them pupate and hatch. The experiments indicate that adenine is more important than guanine and uracil in promoting pupation: larvae do almost as well on adenine alone as on a mixture of adenine, guanine, and uracil. Pupation occurs much later on guanine and uracil, even later than in the absence of any purine or pyrimidine, although eventually a high percentage of the wild type flies on guanine and uracil pupate and hatch. The medium with adenine, guanine, and uracil gives more rapid development and a slightly higher percentage of pupation and hatching than does the original RNA medium.

Because of its structural similarity to the purines, benzimidazole was tried in the medium, either alone or in combination with RNA or adenine (Table IV). A marked difference in the ability of the different stocks to respond to benzimidazole is evident. With RNA or adenine, benzimidazole (1 mg. per ml. of medium) caused the death of wild and Double Bar larvae in 1 or 2 days. The larvae hatched out but were unable to grow. Vestigial larvae were able to grow and survive up to 23 days, but were unable to pupate. On benzimidazole alone (1 mg. per ml. of medium), wild and Double Bar larvae again were unable to survive and grow, whereas vestigial larvae grew and a fraction of them were able to pupate and hatch. The numbers pupating and hatching were very similar to those raised on the guanine-uracil medium, but the time of pupation and hatching was a little less on benzimidazole. On a medium containing a lower concentration of benzimidazole (0.1 mg. per ml. of medium), wild as well as vestigial larvae were able to survive, pupate, and hatch, but vestigial larvae still grew better than the wild type.

DISCUSSION

The results given in Table II show that all of the stocks tested can grow about as well on a medium containing nucleotides from hydrolyzed RNA as on one containing RNA itself. The growth-promoting effect does not reside in some specific arrangement of the components of RNA or in RNA as such, but in the nucleotides themselves and specifically in the purines and pyrimidines. Of the three purines and pyrimidines tested (cytosine, the other pyrimidine found in RNA, was unavailable), adenine seems to be the most important factor in causing rapid growth. Wilson (10), using an inadequate yeast medium, found 0.004 M adenine to be toxic. The fact that the flies are killed by adenine in the absence of sugar but survive on adenine plus sugar shows that adenine itself is not toxic, but perhaps makes some demand on the larva or stimulates larval growth in some way to which the larva is unable to respond in the absence of sugar. In the presence of sugar, adenine causes a marked decrease in the length of the larval period, but the percentage of larvae pupating and hatching is relatively low. The experiments indicate that guanine, in contrast to adenine, causes an increase in the time necessary for development. When adenine and guanine are combined, the time of development is short, about the same as for adenine alone, but a much higher percentage of larvae pupate and hatch. Guanine seems to have some function in regulating the growth-stimulating action of adenine. Schultz *et al.* mentioned in their abstract (7) that desoxyribonucleic acid inhibits the growth of larvae. Our results, with a desoxyribonucleic acid prepared from calf thymus by the Mirsky and Pollister (11) method, corroborate this. Larvae raised on a medium containing 0.3 mg. of desoxyribonucleic acid per ml. of medium (Table III) lived for a long time (up to 28 days), but grew only very slowly, never attained normal size, and died without pupating. The possibility remains that this is due to some impurity present in the desoxyribonucleic acid preparation.

These experiments suggest that the *Drosophila* larva has extensive enzyme systems for converting one type of substance into another. In the experiments with a sugarless medium, the larvae which survived must have converted amino acids or the fatty acids from lecithin into carbohydrates to be utilized for energy. Similarly, the larvae grown on nucleic acid-free media presumably synthesized the purines, pyrimidines, and ribose from simpler compounds and combined them with the phosphate present in the medium to make nucleic acid. The rate of the synthesis of nucleotides is apparently less than enough to supply the optimal amounts for growth, however, since the addition of nucleotides or purines and pyrimidines speeded growth.

Benzimidazole is an inhibitor of the growth-promoting action of RNA or adenine presumably because it is a structural analogue of the purines and has the same relation to them that the sulfonamides have to *p*-aminobenzoic acid. The vestigial stock seems to contain some mutant gene (not necessarily the *vg* gene itself, of course), the biochemical expression of which is to enable this stock to use benzimidazole much better than wild type or Double Bar can.

The appearance of the phenocopy, the alteration of the genetically Double Bar eyes toward wild type, on the medium lacking sugar and nucleic acid may be due to the lengthening of the time of development (21 days from egg to pupation). It has been found that low temperature or a specific genetic modifier of the Bar type (12) will increase the number of eye facets in the *B* or *BB* eye. The increased time of development upsets the relation between the various developmental reactions which normally exist in Bar larvae and results in larger eyes. This non-specific effect could be produced by any factor which increases the length of development at the proper time, genetic modifiers, low temperature, poor culture media, or chemicals such as nipagen.

There is no question that in any of these experiments the limiting factor was the amount of food. On those media which would support growth at all, as many as 80 larvae were successfully raised. Twenty was the largest number used in any experimental tube. The flies hatching out of the larvae grown on experimental media were fertile and in many tubes a second generation of flies was raised.

SUMMARY

1. *Drosophilia* were raised on sterile, chemically defined media in which ribonucleic acid was replaced by nucleotides, purines, or pyrimidines, singly or in combination.

2. Larvae grew as well on nucleotides as on ribonucleic acid. The growth-promoting effect of ribonucleic acid resides not in RNA as such, but in the purines and pyrimidines and especially adenine. Guanine seems to have some function in regulating the growth-stimulating action of adenine. Desoxyribonucleic acid inhibited growth and pupation of the larvae.

3. The "vestigial" stock, in contrast to wild type or Double Bar, was able to survive in the presence of benzimidazole, a structural analogue of the purines, and seems to contain a mutant gene which enables it to utilize benzimidazole in place of the purines.

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GLYCINE, A PRECURSOR OF 5(4)-AMINO-4(5)-IMIDAZOLECARBOXAMIDE

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An *inhibition analysis* (1) of the effect of purines on the toxicity of sulfanilamide for certain bacteria indicated that sulfanilamide prevents *p*-aminobenzoic acid from functioning as a coenzyme, or from being converted to a coenzyme, involved in the biosynthesis of purines. Under certain conditions of sulfonamide inhibition of growth of these bacteria, a precursor of purines might be expected to accumulate in the medium. Such appears to be the case in the accumulation in sulfonamide-containing medium of a non-acetylatable diazotizable amine (2) which has been identified (3) as 5(4)-amino-4(5)-imidazolecarboxamide.

In the present investigation, a study of the effects of amino acids on the quantity of the amine accumulating in the medium of *Escherichia coli* has been made. Only a small amount of the amine is formed in a medium restricted to salts and glucose together with moderate amounts of sulfadiazine; however, in the presence of glycine, *E. coli* produces the amine in proportion to the amount of available glycine. Details of the effects of glycine and other amino acids on the formation of 5(4)-amino-4(5)-imidazolecarboxamide by *E. coli* in the presence of sulfadiazine are given below.

EXPERIMENTAL

Materials—The hydrochloride of glycinamide was prepared from chloroacetamide by the method of Bergell and von Wülfing (4); N-formylglycine was prepared by the action of a mixture of formic acid and acetic anhydride on glycine, as described by Biilmann, Jensen, and Jensen (5).

Testing Method—An inorganic salts-glucose medium was prepared as follows: Na_2SO_4 1 gm., NH_4Cl 1 gm., K_2HPO_4 0.8 gm., $MgSO_4 \cdot 7H_2O$ 80 mg., $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 20 mg., and glucose 2 gm. were dissolved in water and diluted to 100 cc., steamed, and filtered. For tests, 5 cc. of this double strength medium were added to the test materials in 5 cc. of water; the tubes (20 mm. × 10 cm.) were covered with a clean towel, autoclaved 10 minutes at 15 pounds steam pressure, and inoculated. Use of short, wide tubes loosely stoppered or covered with a towel was found to give the maximum yield of amine. The organism was a strain of *Escherichia coli* previously described (6), and was grown in the salts-glucose medium prior to being tested. After 24 hours growth, the cells were centrifuged, washed once with 10 cc. of 0.9 per cent sodium chloride solution, and resuspended in 10 cc. of the saline. The test solutions were inoculated with 0.2 cc. of this suspension. After incubation for 18 hours at 37°, the medium in each tube was assayed for non-acetylatable diazotizable amine by the method of Bratton and Marshall (7) after treatment with 2 per cent acetic anhydride for 30 minutes at room temperature (8, 2). Acetic anhydride (0.02 cc.) was added to 1 cc. of the test solution, and the mixture was shaken and allowed to stand 30 minutes; 4 cc. of 0.2 N sulfuric acid followed by 0.5 cc. of 0.1 per cent sodium nitrite were added, and, after 5 minutes, the excess nitrite was destroyed by the addition of 0.5 cc. of 0.5 per cent ammonium sulfamate. 3 minutes later, 0.5 cc. of 0.1 per cent N-1-naphthylethylene diamine dihydrochloride was added and, after 30 minutes, the color was read on a Klett-Summerson colorimeter with a 540 mμ filter. Synthetic 5(4)-amino-4(5)-imidazolecarboxamide (3) was used as a standard.

Results

In Table I, the effect of glycine, threonine, and glutamic acid on the production of 5(4)-amino-4(5)-imidazolecarboxamide by *Escherichia coli* in the presence of 50 γ per 10 cc. of sulfadiazine is indicated. Addition of glycine to the salts-glucose medium resulted in an increase of amine formation proportional to the dosage of glycine over a large range in concentration. Glutamic acid alone was without appreciable effect, but the presence of glutamic acid in the medium increased appreciably the yield of the amine from glycine. Over the range of concentrations of glycine giving a proportional yield of amine, approximately 35 per cent of the glycine was converted into the amine in the absence of added glutamic acid, while the yield approached 50 per cent of the theoretical in its presence. Other amino acids known to possess labile amino groups, *i.e.* leucine, isoleucine, and valine, gave similar results but were not as active as glutamic acid.

As is indicated in Table I, DL-threonine was only about a tenth as active on a weight basis as glycine. Even after correcting for the difference in molecular weight and for the possibility that only L-threonine might be active, the threonine is less active than glycine. Glutamic acid enhanced the activity of threonine in formation of the amine in a manner analogous to its action with glycine.

In separate tests, all other amino acids including serine were practically inactive in replacing glycine. The hydrochloride of glycinamide as well as N-formylglycine was essentially inactive in increasing production of the amine.

Higher concentrations of sulfadiazine increased the amine production of *Escherichia coli* without addition of glycine or threonine; however, in the presence of either of the amino acids, the higher concentrations of sulfadiazine resulted in a similar increase in production of the amine. The level of 50 γ per 10 cc. of sulfadiazine was the most satisfactory for demonstrating the effect of glycine.

TABLE I

*Effect of Amino Acids on Production of 5(4)-Amino-4(5)-imidazolecarboxamide by Escherichia coli**

Amino acid	Amount	Concentration of product†	
		Without added L-glutamic acid	With added L-glutamic acid (1 mg. per 10 cc.)
	γ per 10 cc.	*γ per 10 cc.*	*γ per 10 cc.*
Glycine	0	4.4	5.0
	10	10.0	12.8
	30	21.1	30.6
	50	37.8	43.9
	75	50.6	65.0
	100	61.1	78.9
	200	68.9	87.8
DL-Threonine	100	9.4	13.9
	300	31.1	41.7
	1000	58.9	83.3

* In the presence of sulfadiazine, 50 γ per 10 cc.

† Determined colorimetrically with synthetic 5(4)-amino-4(5)-imidazolecarboxamide (3) as a standard.

DISCUSSION

The conversion of glycine to uric acid has been demonstrated to occur in pigeons (9) and man (10). Pigeons fed glycine with the carboxyl group containing excess C^{13} excrete uric acid containing relatively large concentrations of C^{13} in carbon atoms of position 4 (9). A human adult male fed glycine containing N^{15} excreted uric acid containing relatively large amounts of N^{15} in the nitrogen atoms of position 7 (10).

Similar studies with pigeons (9, 11) have indicated that the carbon atoms of position 6 may be derived from carbon dioxide, and the carbon atoms of positions 2 and 8 are derived from either the carboxyl of acetate or formate.

In view of these results, one would suppose that glycine is probably utilized in a similar manner in the formation of 5(4)-amino-4(5)-imidazolecarboxamide. Since neither the amide nor the formyl derivative of glycine was active in replacing glycine as a precursor of the amine, it appears that the biosynthesis involves a more complex mechanism, perhaps forming 4-imidazolone which is carboxylated and aminated to form the amine.

The increased yield of amine from glycine in the presence of glutamic acid and to a lesser extent leucine and other amino acids indicates their involvement in the amination reactions leading to the production of the amine from glycine. The nitrogen atoms of glutamic acid and purines have been reported to contain similar amounts of isotopic nitrogen after feeding ammonium citrate containing excess N^{15} (12).

The pronounced activity of threonine in the above tests indicates that this amino acid can be converted into glycine by *Escherichia coli.* The reported biosynthesis of threonine from glycine by yeast (13) leaves some doubt as to which precedes the other in the normal course of biosynthesis of the amino acids.

The inactivity of serine, particularly in view of a reported mutant of *Escherichia coli* (14) which requires either glycine or serine for growth, may possibly arise from the inhibition of its conversion to glycine by the sulfadiazine.

SUMMARY

Only small amounts of 5(4)-amino-4(5)-imidazolecarboxamide are produced by *Escherichia coli* in an inorganic salts-glucose medium containing moderate amounts of sulfadiazine. Addition of glycine or threonine to the medium results in increased amine production proportional to the concentrations of the added amino acid over a relatively large range of concentrations. As glycine is somewhat more active than threonine in the test, it appears that this amino acid is a direct precursor of 5(4)-amino-4(5)-imidazolecarboxamide.

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STREPOGENIN ACTIVITY OF DERIVATIVES OF GLUTAMIC ACID*

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Although the structure of strepogenin is not known, some evidence indicates that this peptide-like growth factor is a derivative of glutamic acid (1, 2). Because the evidence on this point was suggestive rather than conclusive, a number of derivatives of glutamic acid have been synthesized and assayed for strepogenin activity in the growth test with *Lactobacillus casei* (3). Peptide derivatives involving each of the three functional groups of glutamic acid were investigated in an attempt to learn from model compounds whether glutamic acid peptides possessed activity, and, if they did, what type of linkage of this amino acid was most conducive to it. A few compounds in which the substitution was on the amino group were found to have significant strepogenin potency. Clues were thus obtained to indicate something of the nature of strepogenin. Some of this work was influenced by the antagonistic relationship which existed between the toxic aspartic acid-containing peptide lycomarasmin and the growth factor strepogenin as described in a preliminary communication (1).

To aid in arriving at any conclusion as to the nature of strepogenin a number of peptides not containing glutamic acid were tested for activity. All of these were found to be inactive. An aspartic acid derivative identical with one of the active glutamic acid peptides, except that aspartic acid replaced the glutamic acid, was found to be inhibitory of growth, and this toxic action was prevented by small amounts of strepogenin concentrate.

Because some of the desired peptides contained serine combined through its carboxyl group, it was necessary to use the azide method of coupling rather than the acid chloride procedure. In the latter, the alcoholic group of serine would be replaced by Cl. Although Fruton has employed carbobenzoxyserine for the formation of seryl peptides (4), the difficulties connected with carbobenzoxy compounds forced us to use *p*-toluenesulfonyl (tosyl) derivatives instead. Tosylated amino acids were readily prepared and could be converted in excellent yield to tosylated di- and tripeptides. These latter could be converted to the free peptides by reduction with metallic sodium and liquid ammonia according to the conditions of du Vigneaud and Miller (5) or of Bovarnick (6). The principal difficulty was the separation of the final peptide from the sodium salts introduced during the reduction. With aspartic acid derivatives, this was easily done by formation of their barium salts, which were sparingly soluble in methanol, but with the glutamic acid compounds complete separation of sodium salts was troublesome. Nevertheless, this new modification of the methods of peptide synthesis has advantages in ease and safety of operation and in stability of the acylated derivatives, which recommend it for further use.

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* A summary of this work has been published (*Federation Proc.*, **6**, 424 (1947)).

† With the technical assistance of R. A. Brown and A. Holloway. The elementary analyses were performed by Dr. A. Elek.

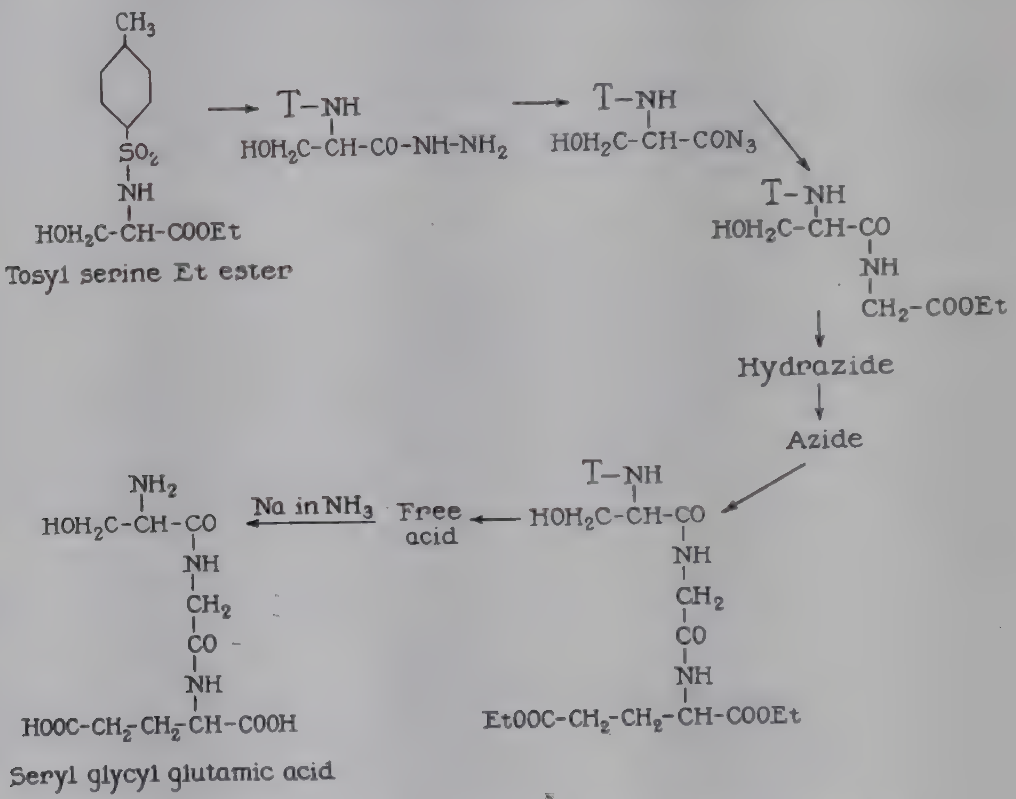

FIG. 1. Reactions in the synthesis of serylglycylglutamic acid

Application to the formation of serylglycylglutamic acid is illustrated by the reactions shown in Fig. 1.

EXPERIMENTAL

Sources of Compounds—Most of the known compounds used in this work were obtained from their originators or were prepared by published methods. Thus, isoglutathione was kindly supplied by Dr. V. du Vigneaud and isoglutamine by Dr. J. S. Fruton. Diglycylglycine, triglycylglycine, glycyltyrosine, tyrosylglycylglycine, leucylleucine, leucylglycylglycine, glycylalanine, glycylleucine, leucyl-α-aminobutyric acid, and α-aminobutyrylleucine were from the collection of Dr. K. Landsteiner. Glutamyltyrosinamide, glutamyltyrosylglutamic acid, prolylglutamic acid, and glycylalanylleucylglutamic acid were kindly supplied by Dr. S. Moore and Dr. W. H. Stein as carbobenzoxy derivatives from the collection of Dr. M. Bergmann. These substances were converted to the free peptides by catalytic hydrogenation in the usual fashion. The acetyl and carbobenzoxy derivatives of glutamic acid and aspartic acid which are listed in a section below were likewise from Dr. Bergmann's collection.

Alanylglycylglutamic acid and glycylalanylglutamic acid were made by coupling α-chloropropionylglycyl chloride and chloroacetylalanyl chloride respectively with glutamic acid, followed by treatment with concentrated aqueous ammonia. The procedures in such syntheses are so well known as not to require detailed description. Analytically pure products were isolated.

Preparation of Serylglycylglutamic Acid

(*a*) *Tosyl-*DL*-serine Hydrazide*—Tosyl-DL-serine was prepared by dissolving 10.5 gm. of DL-serine in 125 cc. of 2 N NaOH, heating to 70°, adding 24 gm. of *p*-toluenesulfonyl chloride, and shaking vigorously until a clear solution was obtained. When this was cooled and acidified with concentrated HCl, 15.1 gm. of tosylserine separated.[1] This manner of tosylating amino acids, first described by Harington and Moggridge (7) for glutamic acid, was found superior to methods involving acylation at room temperature in biphasic solvents and hence was used throughout this work. The tosylserine was converted to the ethyl ester by dissolving it in absolute alcohol saturated with HCl gas, allowing the solution to stand overnight at room temperature, and concentrating to dryness under reduced pressure. The process was then repeated. For preparation of the hydrazide the product was not purified, but was dissolved in 100 cc. of absolute alcohol, and the solution was treated with 8.2 cc. of hydrazine hydrate.[2] The hydrazide began to separate almost immediately as white crystals. After the mixture had stood for 24 hours at room temperature, these were filtered off and washed. Yield 13.4 gm., m.p. 155° after softening from 148°. The compound was insoluble in water but readily dissolved in dilute HCl or NaOH. It was sparingly soluble in ethanol, and could be recrystallized from this solvent.

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[1] The yield of tosylserine was considerably less than that found for tosylglycine, tosylaspartic acid, and tosylglutamic acid. This poor yield of the serine derivative was not due to the solubility of it in the reaction mixture, because extraction of the latter with ethyl acetate yielded only negligible amounts of material. Furthermore, treatment of the filtrate from the tosylserine with more tosyl chloride and alkali yielded no additional tosylserine.

[2] It was necessary to use a large excess of hydrazine hydrate in order to obtain good yields. This was true of all hydrazides studied in this work.

$C_{10}H_{15}O_4N_3S$. Calculated, N 15.4; found, N 15.1

(*b*) *Tosyl*-DL-*serylglycyl Hydrazide*—13.4 gm. of tosylserine hydrazide were dissolved in 125 cc. of cold water plus 4.0 cc. of concentrated HCl. 9 cc. of acetic acid were added to the solution, and the mixture was cooled to 0° and treated with a solution of 5.5 gm. of $NaNO_2$ in 35 cc. of water. The addition was made slowly with stirring. The azide separated as a semisolid, and was immediately extracted with four portions of ice-cold ethyl acetate. The combined extracts were shaken out once with 50 cc. of dilute $NaHCO_3$ solution,[3] and the organic phase was dried in the cold with $MgSO_4$ for an hour. To this dried solution of the azide a dry ether solution of glycine ethyl ester prepared with the usual precautions from 21 gm. of its hydrochloride was added, and the mixture was allowed to stand at room temperature for 24 to 40 hours. The solution was then extracted with a slight excess of 1 N HCl in order to remove unchanged glycine ester, and the organic phase was washed once with water, dried with $MgSO_4$, and concentrated to constant weight under reduced pressure. Yield 15.8 gm.

The crude tosylserylglycine ethyl ester was then dissolved in 100 cc. of absolute alcohol and converted to the hydrazide by addition of 4 cc. of hydrazine hydrate as described in (*a*) for tosylserine hydrazide. Yield 13.7 gm., m.p. 215–216°. The substance was less soluble in ethanol than that described in (*a*) and was therefore best recrystallized by dissolving it in the calculated amount of 1 N NaOH and adding the requisite quantity of HCl.

$C_{12}H_{18}O_5N_4S$. Calculated, N 17.0; found, N 17.0

(*c*) *Tosylserylglycylglutamic Acid*—13.2 gm. of tosylserylglycine hydrazide were dissolved in 125 cc. of water, 5 cc. of concentrated HCl, and 10 cc. of acetic acid and converted to the azide with 4.2 gm. of $NaNO_2$ in a manner similar to that described in (*b*). The ethyl acetate solution of the azide was allowed to react with diethyl glutamate (prepared from 29 gm. of diethyl-L-glutamate hydrochloride) in the same way as described in (*b*). The liquid ester so obtained weighed 21.4 gm.

To prepare the free acid, this ester was dissolved in 200 cc. of methanol and neutralized with methanolic 1 N NaOH. 22 cc. of aqueous 4 N NaOH were added, and, after half an hour at room temperature, the solution was acidified with a slight excess of 1 N HCl. The mixture was concentrated under reduced pressure to about 30 cc., at which point an oil had begun to separate. This was dissolved by adding 50 cc. of water, and the solution was extracted six times with 100 cc. portions of ethyl acetate. The extracts were dried with $MgSO_4$ and the solvent was evaporated under reduced pressure. A hygroscopic solid weighing 12.3 gm. was thus obtained which was best recrystallized by dissolving in a few cc. of chloroform and adding dry ether. From ethyl acetate-ether or alcohol-ether somewhat sticky products resulted.

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[3] Since the azide contained the acidic sulfonamide group, sufficient $NaHCO_3$ to extract the compound as the salt was not used.

$C_{17}H_{23}O_9N_3S$. Calculated, N 9.4; found, N 9.6

Analysis of the product was difficult because it was quite hygroscopic. It was undoubtedly a mixture of two diastereomers since racemic serine and L-glutamic acid had been used in its preparation.

(*d*) *Serylglycylglutamic Acid*—5.9 gm. of tosylserylglycylglutamic acid were placed in a 300 cc. 3-necked round bottom flask equipped with a mechanical stirrer and a soda lime guard tube. This flask was immersed in an alcohol and solid CO_2 bath and 100 cc. of liquid ammonia were added through a long, wide glass tube placed in the third neck of the flask. Stirring was begun, and when solution was complete, 3.0 gm. of metallic sodium cut in small pieces were added slowly. Stirring was continued for 4 hours, and then 6.5 gm. of ammonium chloride were added. The motor was stopped when all the blue color had been discharged, the cooling bath was removed, and the NH_3 was allowed to distil off. The flask and its contents were then placed in a vacuum desiccator over concentrated sulfuric acid overnight. The reaction product was then dissolved in 100 cc. of 0.2 N H_2SO_4 (final pH 3.0 to 4.0) and the thiocresol removed by four extractions with ether. The aqueous phase was freed of NH_3 by adding $Ba(OH)_2$ solution to it until it was just pink to phenolphthalein. It was then concentrated under reduced pressure at low temperature, and as the pH fell more $Ba(OH)_2$ was added to maintain the mixture constantly pink to phenolphthalein. The solution was freed of barium with H_2SO_4 exactly. The pH was adjusted to 4.0 with NaOH, the $BaSO_4$ was removed by filtration, and the resulting solution was evaporated under reduced pressure to a syrup. The peptide was precipitated together with some NaCl by addition of 200 cc. of absolute alcohol. This precipitate was separated, dried, and dissolved in water, and chloride ion was removed with silver acetate and silver with H_2S. The resulting filtrate was adjusted to pH 4.0 with HI and evaporated under reduced pressure to a syrup from which the peptide was precipitated with 200 cc. of absolute alcohol. The material thus obtained, when dried, weighed 2 gm. and consisted of serylglycylglutamic acid contaminated with some ash. In order to prepare the pure peptide, this substance (600 mg.) was suspended in absolute alcohol (30 cc.) and concentrated HI (1.0 cc.). After a few minutes the peptide had dissolved as the hydroiodide and could be separated from insoluble impurities. It was then precipitated by adjusting the alcoholic solution to pH 4.0 with concentrated NH_4OH. The product thus obtained was dried and extracted with glacial acetic acid. When this extract was poured into 10 volumes of absolute alcohol, pure serylglycylglutamic acid was precipitated.

$C_{10}H_{17}O_7N_3$. Calculated, N 14.4; found, N 14.6

Several attempts were made to work up the reduction product by concentrating the aqueous solution (after ether extraction of thiocresol) to dryness, followed by extraction with alcohol plus HCl. The peptide was then precipitated from the solution so obtained by adjusting to pH 4 with ammonia or pyridine. The yields by this method never exceeded 40 per cent and the product was never secured free of ash.

Bioassay of the crude reaction mixture in comparison with the pure peptide showed that the reduction to serylglycylglutamic acid was practically quantitative. The low yield must, therefore, have been due to losses during purification.

Glycylserylglutamic Acid—The stages in the synthesis were the same as those described for serylglycylglutamic acid except that tosylglycine was the starting material. Tosylglycine hydrazide melted at 160° and contained 17.3 per cent N (theory, 17.3). Tosylglycyl-DL-serine hydrazide melted at 180° and contained 16.1 per cent N (theory for $C_{12}H_{18}O_5N_4S \cdot H_2O$, 16.1). Tosylglycylserylglutamic acid, hygroscopic crystals from chloroform and ether, contained 9.3 per cent N (theory, 9.4), but it was probably a diastereomeric mixture as was the isomer described above.

Serylglycylaspartic Acid—Tosylserylglycine hydrazide was converted to the azide and coupled with diethyl L-aspartate in a fashion similar to that used for the glutamic acid compound. After hydrolysis of the ester, the yield of free acid melting at 100–105° was 94 per cent and was found to contain 9.4 per cent N (theory, 9.7). After reduction with sodium and liquid ammonia, the reaction mixture was dissolved in hydrochloric acid at pH 4 and thiocresol was removed with ether. The aqueous solution was then brought to pH 8 with methanolic barium hydroxide, and enough methanol was added to give a final concentration of 80 per cent. After the mixture had stood in the cold, the insoluble barium salt was filtered off, washed with methanol, dissolved in water, and freed of barium exactly with sulfuric acid. Chloride was removed from the resulting solution with excess silver acetate and silver was taken out as the sulfide. The resulting filtrate was concentrated under reduced pressure to a small volume and treated with alcohol. Serylglycylaspartic acid was thus obtained in 45 per cent yield based on the weight of its tosyl derivative.

$C_9H_{15}O_7N_3$. Calculated, N 15.2; found, N 14.9

Method of Biological Assay—The compounds were tested for strepogenin activity for *Lactobacillus casei* according to the method of Sprince and Woolley (3, 8). The basal medium was fortified with synthetic pteroylglutamic acid,[4] 0.01 γ per cc., instead of the concentrate of folic acid. In addition, pyridoxamine, 0.1 γ per cc., was also added to the medium.

Each substance was tested in graded doses at concentrations of 1 mg. to 1 γ per cc. of final medium, and the potency was established by comparison of growth responses to those elicited by the preparation of standard liver extract as described previously (3, 8). In this way the activity of the compounds after they had been autoclaved in the basal medium was determined. In addition, parallel assays were performed in which solutions of the substances sterilized by filtration were added to the basal medium after the latter had been autoclaved. In this way the potency of the unheated substances was judged, and, as will appear shortly, this value was greater for some types of peptides than was that found for the heated samples.

Strepogenin Activity of Non-Glutamic Acid Peptides—A considerable number of peptides not containing glutamic acid were assayed in the manner described, and none was found to possess potency. Those tested were alanylglycylglycine, tyrosylglycylglycine, acetyldehydrophenylalanylaspartic acid, carbobenzoxyasparagine, diglycylglycine, triglycylglycine, glycyltyrosine, leucylleucine, leucylglycylglycine, glycylalanine, glycylleucine, leucyl-α-aminobutyric acid, α-aminobutyrylleucine, p-aminobenzoylglycine, glycyl-p-aminobenzoic acid, serylglycylaspartic acid, and asparagine.

Strepogenin Activity of Derivatives of Glutamic Acid—Table I shows the relative strepogenin potency of a variety of peptides containing glutamic acid. Glutamine, glutathione, glutamyltyrosylglutamic acid, serylglycylglutamic acid, glycylalanylglutamic acid, alanylglycylglutamic acid, glycylserylglutamic acid, and glycylglutamic acid all showed some potency. The other derivatives were inactive. The need for a free amino group (*cf.* (2, 3, 8)) was illustrated by the finding that tosylserylglycylglutamic acid and chloroacetylalanylglutamic acid were inactive, whereas the corresponding peptides with free amino groups possessed potency.

Isoglutamine and isoglutathione, both derivatives involving the α-carboxyl group of glutamic acid, showed some activity. However, since it is well known that these substances may be contaminated with the isomeric glutamine and glutathione respectively, it was probable that they owed their activity solely to these impurities. This appeared even more probable when it was found that the growth-promoting power they did possess was destroyed by heat (*cf.* the next section). Isoglutamine and isoglutathione are not destroyed by boiling their aqueous solutions, whereas glutamine and glutathione are.

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[4] This material was kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company.

Differentiation of Active Compounds by Heat Stability—The data in Table I show that the only compounds which were not inactivated by heating in the basal medium were those in which the substituent was on the amino group of the glutamic acid, *e.g.* serylglycylglutamic or alanylglycylglutamic acid. Active substances such as glutamine, which bore the substituents on the γ-carboxyl group of glutamic acid, were inactivated wholly or in part by autoclaving them in the basal medium. The potency of glutamine was destroyed completely when it was heated by itself in aqueous solution and then added to the sterilized basal medium, but when it was autoclaved in the basal medium about 1 per cent of its activity remained. For comparison with these results, the potency of strepogenin present in tissue extracts or in tryptic digests of pure proteins was found to be unaffected by autoclaving in the basal medium.

TABLE I

Relative Strepogenin Acitivity of Derivatives of Glutamic Acid

Peptide		Relative strepogenin potency*	
		Before heating	After heating
Derivatives of γ-COOH	Glutamine	50–200†	0
	Glutathione	4–10†	1–3
Derivatives of α-COOH	Isoglutamine	1–3	0
	Isoglutathione	1–5	0
	Glutamyltyrosine ethyl ester	0	0
	Glutamyltyrosinamide	Trace	0
	Glutamyltyrosylglutamic acid	0.8‡	0.8‡
Derivatives of NH_2	Serylglycylglutamic acid	1	1
	Glycylserylglutamic "	0.4	0.4
	Alanylglycylglutamic "	0.3	0.3
	Glycylalanylglutamic "	0.3	0.3
	Glycylalanylleucylglutamic acid	0	0
	Glycylglutamic acid	0.1	0.1
	Prolylglutamic "	0	0
	Acetyldehydrophenylalanylglutamic acid	0	0
	Acetylphenylalanylglutamic acid	0	0
	p-Aminobenzoylglutamic acid	0	0

* Relative to liver standard of potency 1.

† Dependent on dosage level, as discussed in the text.

‡ Inhibitory above 300 γ per cc. One glutamic acid is substituted on the α-carboxyl group and the other on the amino group.

Differences in Shape of Response Curves Obtained with Glutamic Acid

Derivatives—Although glutamine possessed considerable strepogenin potency for *Lactobacillus casei*, the shape of the dose-response curve obtained with it was quite different from that typical for strepogenin. This fact is illustrated in Fig. 2, where it can be seen that the curve with glutamine rises more steeply and attains a maximum considerably below that found for the liver extract standard. Thus, with glutamine, maximal growth cannot be achieved. In this respect, the response produced by glutathione was intermediate between the extremes presented by glutamine and liver extract. Because of these qualitative differences in the shape of the response curves, only approximate values could be established for the activity of glutamine and glutathione (*cf.* Table I). Potency obviously depended on which portion of the curves was used for comparison. In contrast to this qualitatively differing activity of derivatives of the γ-carboxyl group of glutamic acid, those peptides which were produced from glutamic acid by substitution on the amino group showed response curves identical in shape with those found for liver extract. Thus, with serylglycylglutamic acid the response curve was superimposable on the standard curve over the range used for assay.

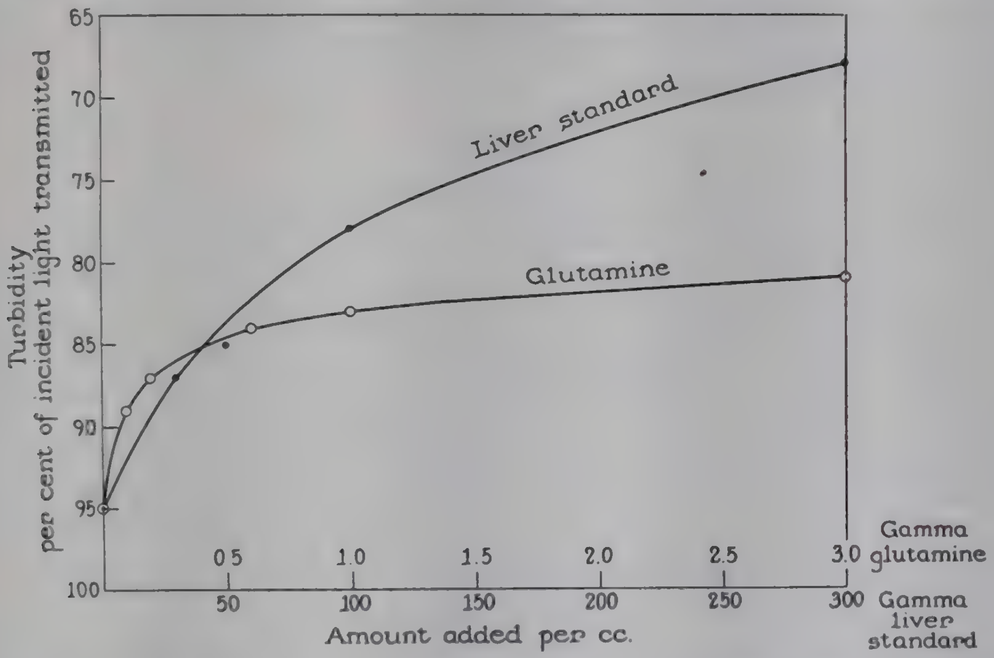

FIG. 2. Growth response curves of *Lactobacillus casei* to liver Fraction L and to glutamine.

Inhibition of Growth Caused by Serylglycylaspartic Acid—Not only was serylglycylaspartic acid inactive, but it was antagonistic to strepogenin. In order to demonstrate this, the basal medium was supplemented with 100 γ of liver Fraction L (standard for strepogenin assays) per cc. In the absence of the serylglycylaspartic acid, fair growth of *Lactobacillus casei* occurred in such a medium, but when 1 mg. of the peptide per cc. was added, multiplication was reduced to two-thirds of the former value. When liver Fraction L was increased to 200 γ per cc., serylglycylaspartic acid was no longer inhibitory.

Although inhibition of growth could be produced by serylglycylaspartic acid as outlined in the preceding paragraph, sufficient increase in the concentration of the peptide in such a system resulted in the manifestation of some growth-promoting action. Thus, when 5 mg. of the peptide per cc. were used, good growth was obtained. A similar situation has been noted with antagonistic structural analogues of thyroxine (9) and of pantothenic acid (10, 11) in which sufficient increase in the concentration of the antagonist permitted the demonstration of hormone or vitamin action rather than inhibitory behavior.

DISCUSSION

Some idea of the structural requirements for strepogenin activity may be gleaned from the data presented. In the first place, the only active compounds thus far encountered have contained glutamic acid. This observation adds to the existing evidence that strepogenin is a peptide or peptide-like compound containing glutamic acid. Secondly, activity qualitatively similar to that of strepogenin on *Lactobacillus casei* was found only in derivatives of glutamic acid in which the added substituents were attached to the amino group. Some potency was displayed by compounds formed by substitution of the γ-carboxyl group of glutamic acid (*e.g.* glutamine and glutathione), but qualitative differences in activity from natural strepogenin were found with these. Since such differences were less with glutathione than with glutamine, the possibility remains that in strepogenin there are substituents on the γ-carboxyl group of glutamic acid. Nevertheless, both glutamine and glutathione had no strepogenin activity for hemolytic streptococcus X40 (3, 12), and glutamine likewise was ineffective in replacing strepogenin for mice (13). It would seem that *Lactobacillus casei* was a less exacting organism than these and that a less specific structure was required for it to respond.

Although several peptides of glutamic acid possessed strepogenin potency, some specificity of action in relation to structure could be discerned. Thus, although all four tripeptides, *viz.* serylglycylglutamic acid, glycylserylglutamic acid, alanylglycylglutamic acid, and glycylalanylglutamic acid, had some degree of activity, the mere addition of 1 more amino acid residue, as in glycylalanylleucylglutamic acid, rendered the compound inactive. More than just a glutamic acid peptide was required.

Although synthetic peptides such as serylglycylglutamic acid had strepogenin activity qualitatively the same as that of the natural growth factor for *Lactobacillus casei*, they did not approach quantitatively the natural substance. For example, the most active compound described in this paper (*viz*. serylglycylglutamic acid) had a potency of 1, while concentrates have been prepared in this laboratory from casein and from insulin with a potency of 40. At best, the present compounds can only serve as models with which to study the nature of strepogenin.

SUMMARY

Tosylated amino acids have been used to prepare a number of tosylated peptides, and these have been reduced with sodium in liquid ammonia to yield the free peptides. In this way serylglycylglutamic acid, serylglycylaspartic acid, and glycylserylglutamic acid have been prepared for the first time. A large number of peptides have been tested for their ability to replace strepogenin in the growth of *Lactobacillus casei*. Only certain derivatives of glutamic acid were found active. True strepogenin potency was observed only in serylglycylglutamic acid, glycylserylglutamic acid, alanylglycylglutamic acid, glycylalanylglutamic acid, and glycylglutamic acid. Several other glutamic acid derivatives in which the amino group was substituted were inactive. Glutamine and glutathione had potency, but this could be distinguished qualitatively from that of strepogenin or of the foregoing synthetic peptides, and was considered not to be true strepogenin activity. Serylglycylaspartic acid was antagonistic to growth promoted by strepogenin. These facts were discussed in relation to the chemical nature of strepogenin.

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THE CHEMISTRY OF MELANIN

III. MECHANISM OF THE OXIDATION OF DIHYDROXYPHENYLALANINE BY TYROSINASE*

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When 3,4-dihydroxyphenylalanine is oxidized in the presence of phenol oxidases (2, 3) or by silver oxide (4), a red pigment is formed. Enzymically, this pigment is converted to a melanin. In the absence of enzyme, it has been shown that the pigment may rearrange to either 2-carboxy-5,6-dihydroxyindole or to 5,6-dihydroxyindole (5). The structure of the red pigment has therefore been inferred to be either 2-carboxy-2,3-dihydroindole-5,6-quinone or the tautomeric 2-carboxy-2,3-dihydro-6-hydroxyindole-1,5-quinonimine (5–7), and on this basis Raper has proposed the accompanying hypothesis for the *enzymic* conversion of 3,4-dihydroxyphenylalanine to synthetic dopa melanin (5, 6).

This study was undertaken to determine whether or not the postulated reactions beyond the formation of the red pigment do take place in the presence of oxygen and enzyme. The enzymic oxidation of 3,4-dihydroxyphenylalanine was followed spectrophotometrically. The process was observed to proceed in three chromophoric phases, the first corresponding to the formation of the red pigment, the second to an intermediate purple pigment, and the third to the formation of melanin. By comparison of the observed spectra with those of known substances, it was possible to show that a rearrangement of the red pigment does occur during the enzymic oxidation, that synthetic dihydroxyphenylalanine melanin is probably a polymer of indole-5,6-quinone, and that the inferred *o*-quinonoid formulation of the red pigment is correct.

EXPERIMENTAL

The technique employed for observing the spectrophotometric course of the enzymic oxidation of dihydroxyphenylalanine has been described (1). A previously described tyrosinase preparation was used (1, *cf.* 8, 9). Silver oxide was prepared according to the directions of Helferich and Klein (10). 3,4-Dihydroxyphenyl-L-alanine was obtained from the Hoffmann-La Roche Company. It melted at 272–278° (total immersion);

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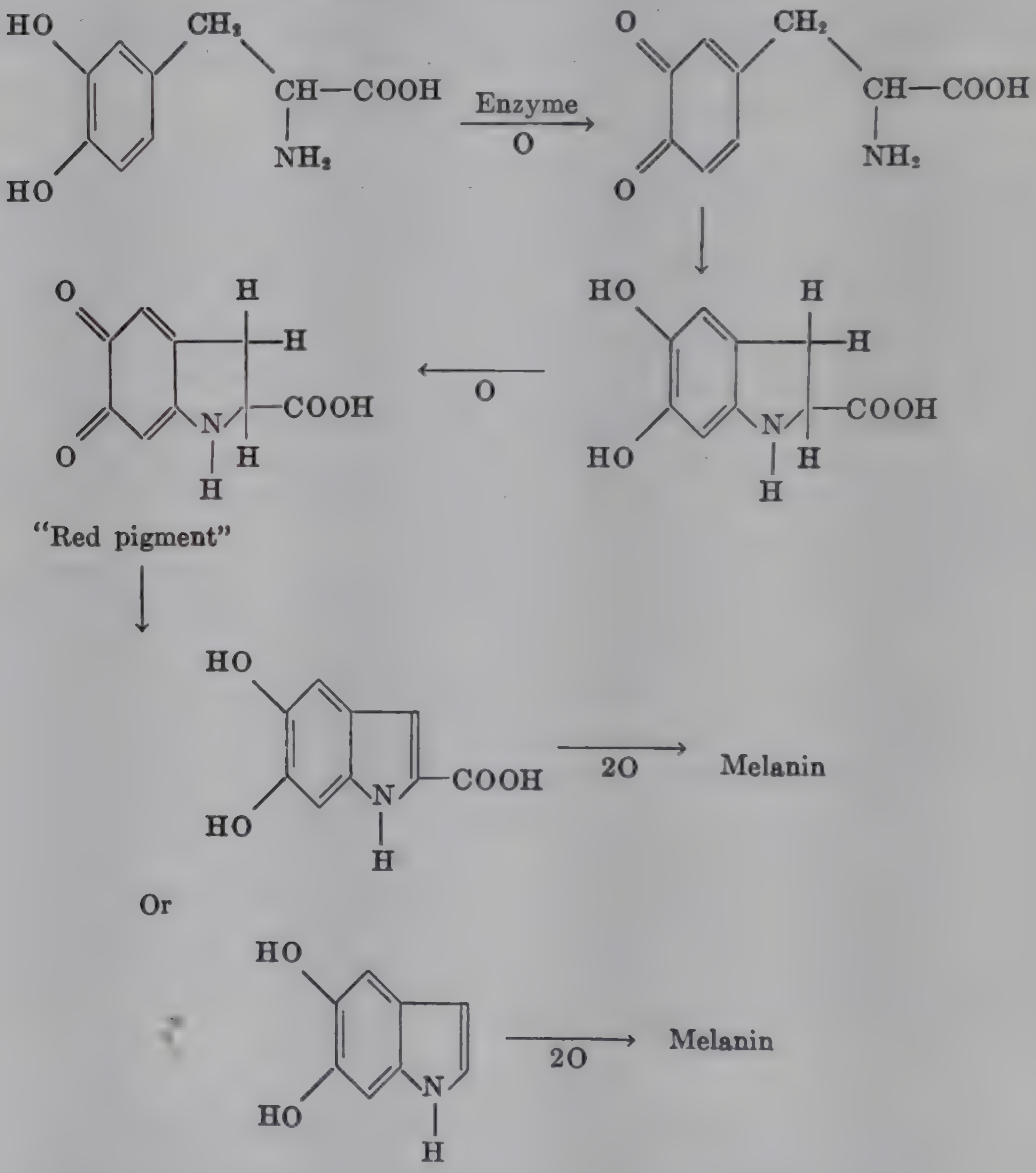
* For Paper II in this series, see Mason (1).

$[\alpha]^{20}_{D} = -11.7°$ in 1.0 N HCl (micro polarimeter tube). Buffers were prepared by adding 0.1 N NaOH to 50 ml. of 0.1 M KH_2PO_4 and diluting the mixture to 100 ml. All the experiments were conducted at temperatures of 25–28°.

Enzymic Formation of Melanin (*Raper*)



2-Carboxy-5,6-dimethoxyindole was synthesized from 6-nitrohomoveratrole by the procedure of Oxford and Raper (11). The product crystallized from acetone-benzene in tablets which melted at 208°. It has been reported to melt at 202–203° (11). The compound was further identified by elementary analysis.

Analysis[1]—$C_{11}H_{11}O_4N$. Calculated, C 59.7, H 5.02, N 6.34
Found, " 59.7, " 5.24, " 6.40

5,6-Dimethoxyindole was obtained from Professor H. S. Raper, whose kindness is acknowledged. The sample melted at 152–154°.

In this report optical density $D = \epsilon ml$, where l is 1.0 cm. and m is the given concentration of the absorbing substance in moles per liter. The molecular extinction coefficient ϵ corresponds to the calculated optical density of a 1 M solution.

Silver Oxide Oxidation of 3,4-Dihydroxyphenylalanine—The reaction between 50 mg. of silver oxide and 10 ml. of a solution buffered at pH 5.6 and containing 0.027 mg. (1.37×10^{-7} mole) of 3,4-dihydroxyphenylalanine per ml. was allowed to continue for periods varying from 0.5 to 10 minutes. In these experiments the reagents were shaken together for the designated period and the resultant solution filtered through No. 42 Whatman paper. Absorption spectra were then immediately measured. The results are depicted in Fig. 1, *A* and *B*. In the course of the reaction, the absorption maximum of 3,4-dihydroxyphenylalanine at 280 mμ was replaced by maxima at 305 and 475 mμ. The intensities of these maxima increased as the reaction period was increased to 3 to 4 minutes, then gradually decreased. Absorption spectra of substances intermediate between 3,4-dihydroxyphenylalanine and the red pigment did not become evident. The solvent buffer alone was shaken with silver oxide in the proportions described for 5 minutes. No change in the absorption spectrum was observed.

Rearrangement of Red Pigment—To determine the spectrochemical characteristics of the substances formed when the pigment rearranges, solutions were prepared by shaking together the reagents described above for 3 minutes, then filtering. Dissolved oxygen was rapidly removed by repeatedly exhausting the reaction vessel at an oil pump. After the solution had stood overnight, all color was discharged, although a small amount of black solid separated. This was removed on No. 42 Whatman paper and discarded. In the absorption spectrum of the filtered solution were found sharply defined maxima at 275 and 298 mμ, as are depicted in Fig. 2, *A*, Curve 2.

The red pigment was also observed to decolorize in the presence of acid. If a solution, prepared by the silver oxide reaction at pH 5.6, was adjusted to pH 1.3 with hydrochloric acid, decolorization was complete in 20 minutes. At pH 1.6 this process took 40 minutes and at pH 2.0 approximately 60 minutes. The absorption spectrum of the product formed at these pH levels displayed a strong maximum at 310 mμ (Fig. 2, *A*, Curve 3) which did not shift upon neutralization to pH 5.6. Solutions of the acid rearrangement product were also adjusted to pH 3.5 and 5.6 and allowed to stand 24 hours in the absence of oxygen. Small amounts of black pigment separated and were removed. The filtered solutions possessed the same absorption spectrum as the freshly prepared rearrangement product, although the intensities of the maximum at 310 mμ were lowered as much as one-third.

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[1] I am indebted to Charles Kinser, National Institute of Health, for this microchemical analysis.

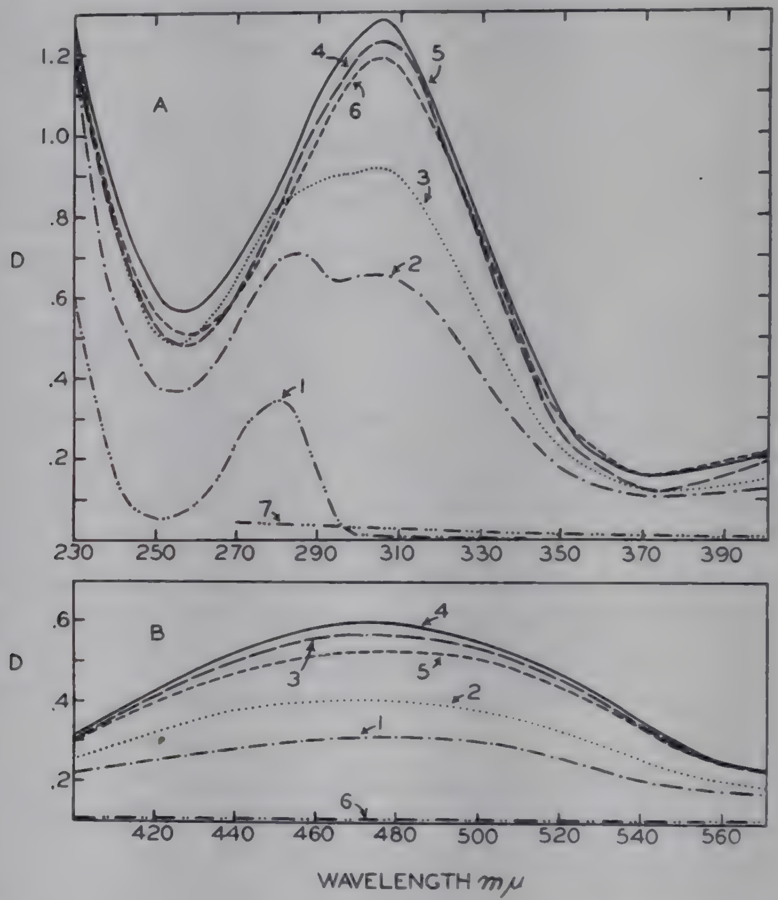


FIG. 1. The spectrophotometric course of the reaction between silver oxide and 3,4-dihydroxyphenylalanine. *A*, Curve 1 represents the spectrum of the original 3,4-dihydroxyphenylalanine solution, Curve 2 after 0.5 minute of reaction, Curve 3, 1 minute, Curve 4, 2 minutes, Curve 5, 3 and 4 minutes, Curve 6, 10 minutes, and Curve 7, 5 minutes of reaction between silver oxide and buffer alone. *B*, Curve 1 represents the spectrum observed after 0.5 minute of reaction, Curve 2, 1 minute, Curve 3, 2 minutes, Curve 4, 3 and 4 minutes, Curve 5, 10 minutes, and Curve 6, 5 minutes of reaction between silver oxide and buffer alone.

The rearrangement product giving the absorption spectrum with maxima at 275 and 298 mμ was found to be readily oxidized either by silver oxide or in the presence of tyrosinase and oxygen. In order to obtain the absorption spectrum of an intermediate product of oxidation, it proved necessary to conduct the reaction in the presence of a limited amount of enzyme, 6.5 units in 0.1 ml. added to 3.0 ml. of solution. This reaction was carried out at pH 5.6 and 6.8. In each case a purple color developed and maxima appeared in the absorption spectrum at 300 and at 540 mμ. These changes are illustrated in Fig. 2, *B*, Curves 2 and 3.

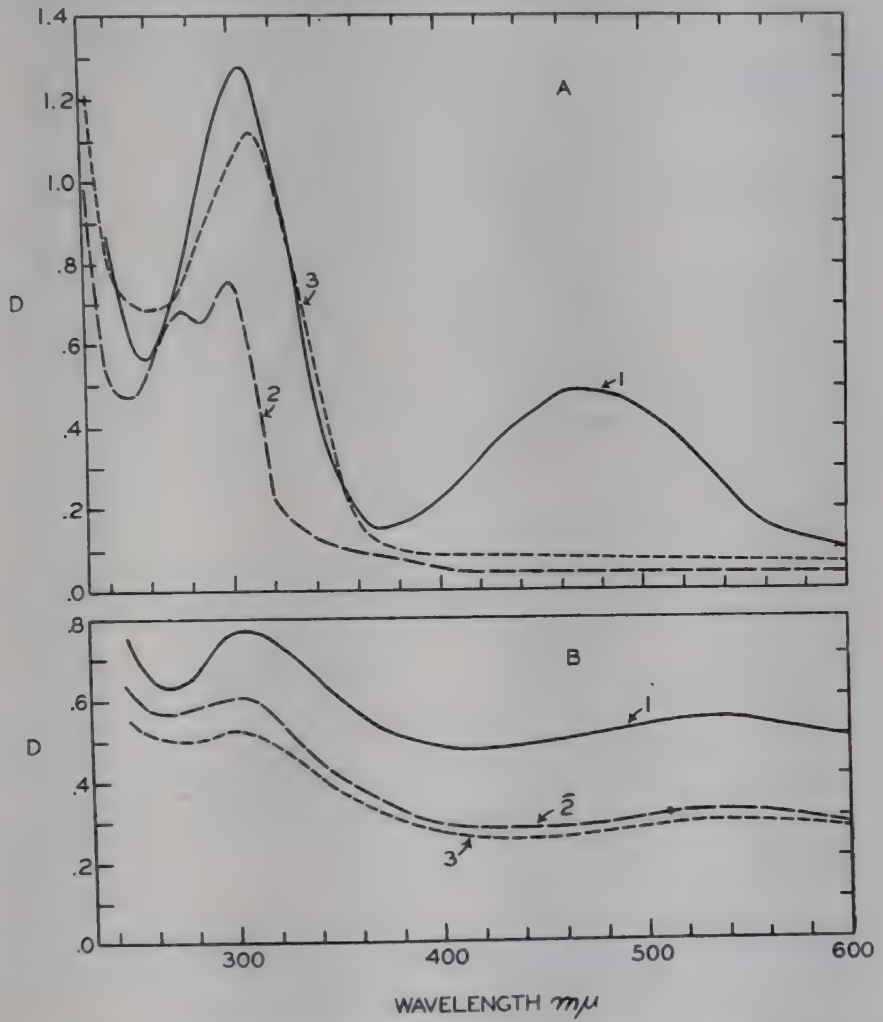


FIG. 2. Absorption spectra of intermediate oxidation products of 3,4-dihydroxyphenylalanine. *A*, Curve 1 represents the optimal spectrum obtained during the silver oxide-3,4-dihydroxyphenylalanine reaction, Curve 2 that observed 24 hours later, oxygen excluded, and Curve 3 that observed 20 minutes after the red pigment solution was brought to pH 1.3. *B*, Curve 1 represents the spectrum observed after 150 minutes of reaction between 3,4-dihydroxyphenylalanine and tyrosinase, Curve 2 that after 20 minutes of reaction between 5,6-dihydroxyindole (prepared stepwise from 3,4-dihydroxyphenylalanine) and tyrosinase at pH 5.6, and Curve 3 after 15 minutes reaction between the same reagents at pH 6.8.

Enzymic Oxidation of 3,4-Dihydroxyphenylalanine—The concentration of 3,4-dihydroxyphenylalanine solutions employed in this study was maintained at 0.027 mg. per ml. To 3.0 ml. of substrate solution were added

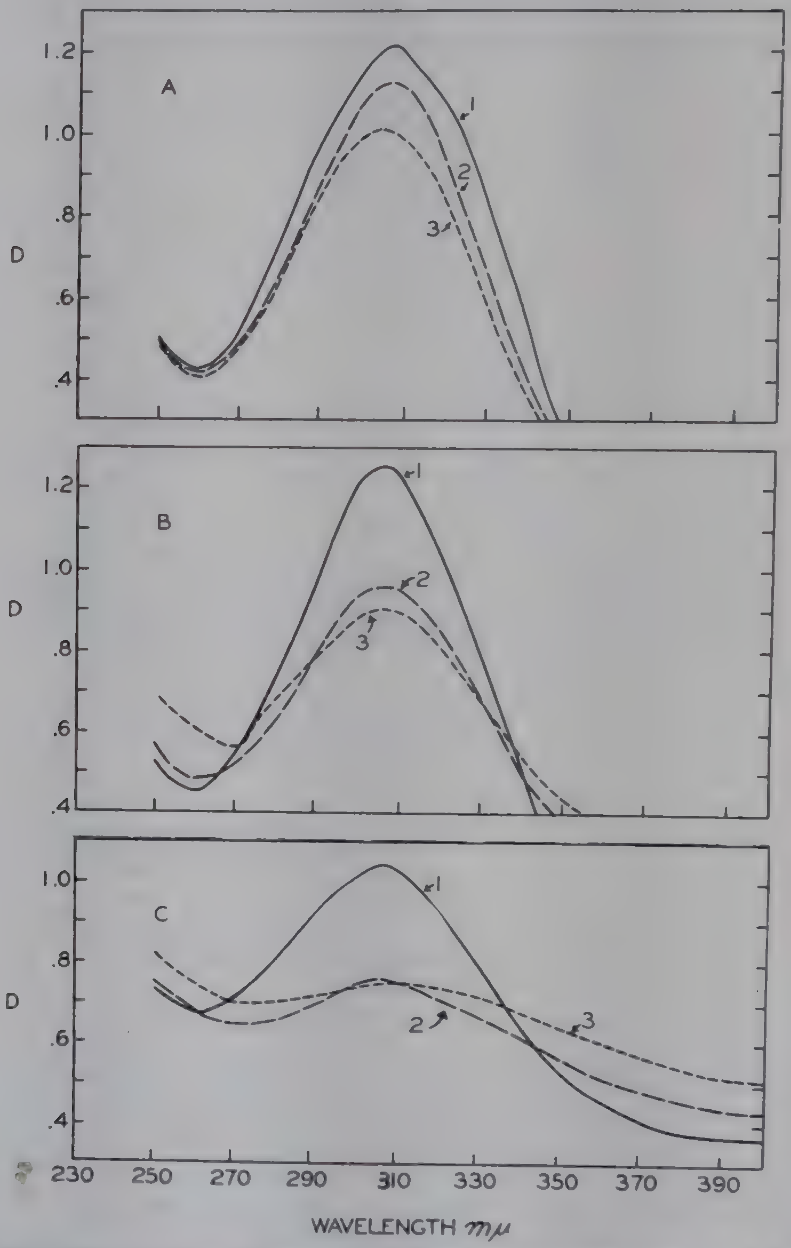


FIG. 3. The spectrophotometric course of the enzymic oxidation of 3,4-dihydroxyphenylalanine. The curves represent the spectra observed, *A*, after 10 minutes, *B*, after 25 minutes, and *C*, after 100 minutes of reaction at (Curve 1) pH 5.6, (Curve 2) pH 6.8, and (Curve 3) pH 8.4.

27 units of tyrosinase in 0.1 ml. The experiments were conducted at pH 5.6, 6.8, and 8.4. In Fig. 3, *A*, *B*, and *C* are presented the absorption spectra obtained after 10, 25, and 100 minutes of reaction. The rates of

change of density at 305 mμ at the three pH values were also determined (25°) and the results are presented in Fig. 4. The absorption spectrum of the pigment obtained in this manner was the same as that prepared by the silver oxide reaction. No variation in the position of the maxima as a result of differences in pH was observed.

After some minutes of reaction general absorption became apparent in the spectra measured at each pH. However, at 150 minutes (in the presence of tyrosinase) at pH 6.8, an intermediate phase showing maxima at 300 and at 540 mμ developed. This is illustrated in Fig. 2, *B*, Curve 1. The absorption spectra of the reaction mixtures at each pH after 24 hours are depicted in Fig. 5.

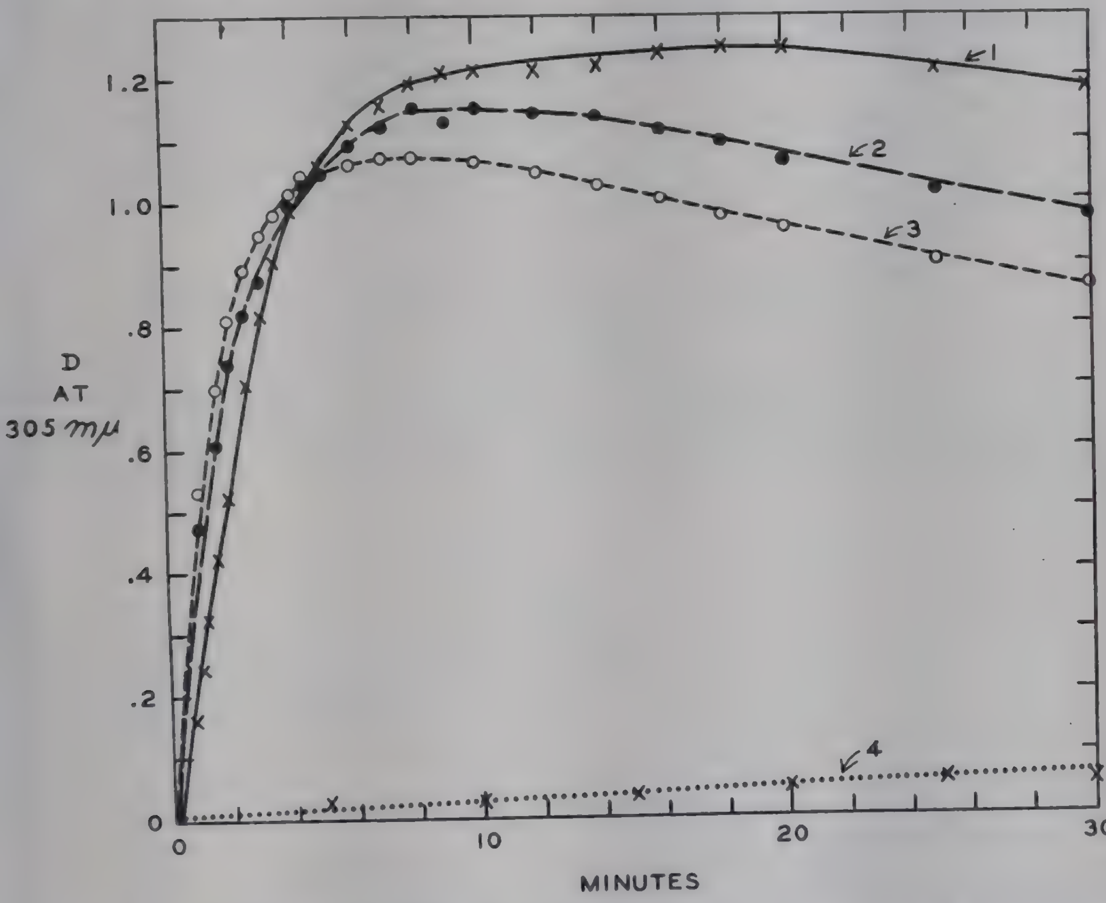


FIG. 4. The spectrophotometric course of the enzymic oxidation of 3,4-dihydroxyphenylalanine. The curves represent the optical density at 305 mμ as a function of time when the reaction was conducted at (Curve 1) pH 5.6, (Curve 2) pH 6.8, and (Curve 3) pH 8.4. Curve 4 represents optical changes in the substrate solution at pH 8.4 in the absence of enzyme.

The absorption spectra of 3,4-dihydroxyphenylalanine and the red pigment (silver oxide preparation) at pH 5.6 have been recalculated as

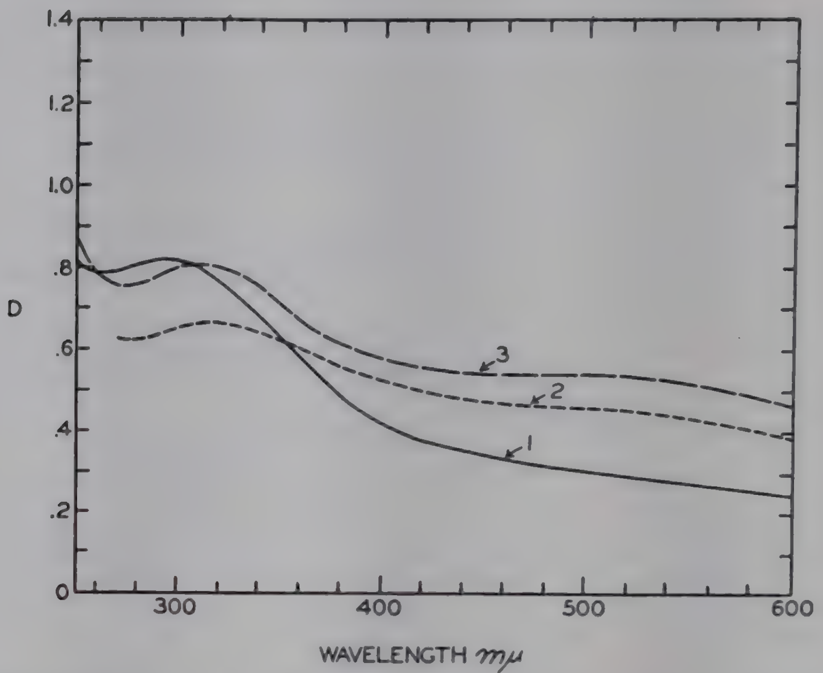


FIG. 5. Spectral curves of synthetic 3,4-dihydroxyphenylalanine melanins. The curves represent the spectra observed after 24 hours of reaction between 3,4-dihydroxyphenylalanine and tyrosinase at (Curve 1) pH 5.6, (Curve 2) pH 6.8, and (Curve 3) pH 8.4.

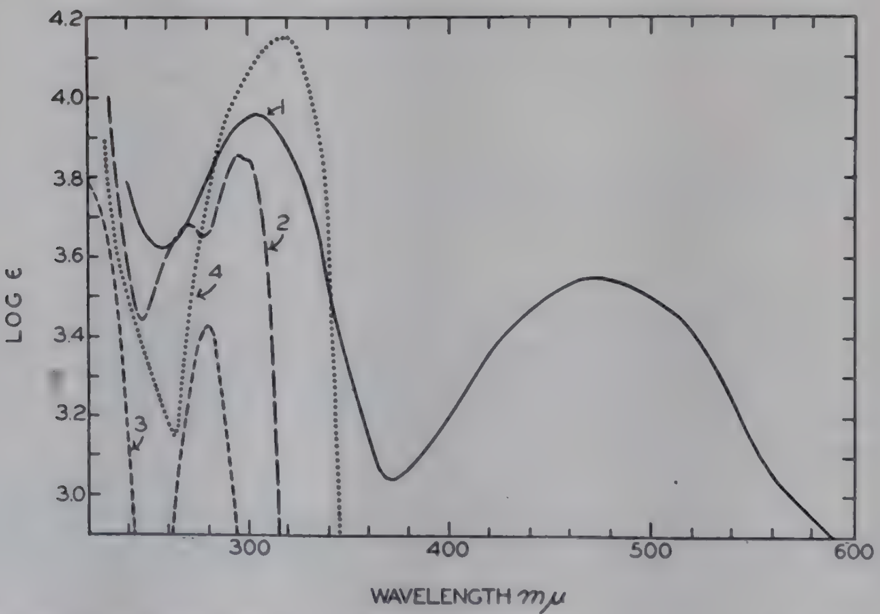


FIG. 6. Molecular extinction coefficients of (Curve 1) 2-carboxy-2,3-dihydroindole-5,6-quinone in buffer, pH 5.6 (silver oxide preparation), (Curve 2) 5,6-dimethoxyindole in ethanol, (Curve 3) 3,4-dihydroxyphenylalanine in buffer, pH 5.6, and (Curve 4) 2-carboxy-5,6-dimethoxyindole in ethanol.

nolecular extinction coefficients and are presented in Fig. 6. To these nave been added the absorption spectra of 5,6-dimethoxyindole and 2-carboxy-5,6-dimethoxyindole in ethanol.

DISCUSSION

The spectral changes accompanying the transformation of tyrosine or 3,4-dihydroxyphenylalanine into synthetic melanins have previously been studied by Bloch and Schaaf (12), Florence, Enselme, and Pozzi (13), and by Ginsburg (14). Absorption spectra characteristic of intermediate substances were not reported.

The absorption spectrum of 3,4-dihydroxyphenylalanine has been determined by a number of investigators. The values reported are summarized in Table I. In the present study, the absorption spectrum of 3,4-dihydroxyphenylalanine at pH 5.6 and 6.8, maximum = 280 mμ, log ε = 3.43 (Fig. 6), did not show an inherent dependence upon hydrogen ion concentration. In view of the relative agreement between the values found and those reported by de Gouveia, Abderhalden, and Marchlewski, the values of Bloch, Schaaf, and Henri, and Florence and Bessières may reflect autoxidative degradation of the solute and the presence of substances other than 3,4-dihydroxyphenylalanine in solution. Under these circumstances, reversibility of the apparent pH effect is improbable.

TABLE I

Absorption Spectrum of 3,4-Dihydroxyphenylalanine

Bibliographic reference	Solvent	Maximum	Log ε
		mμ	
Bloch and Schaaf (12)	0.1 N $NaHCO_3$, pH 8.1	280.7	4.26
Florence and Bessières (15)	pH 1.0	282	4.09
	pH 11.6	303	4.61
de Gouveia, Coelho, and Schon (16)	0.1 N HCl	279.8	3.41
	0.1 N NaOH	298.3	3.60
Abderhalden and Rossner (17)	Water	278	3.39
Marchlewski and Skarzynski (18)	0.1 N HCl	280.8	3.42
Present investigation	pH 5.6–6.8	280	3.43

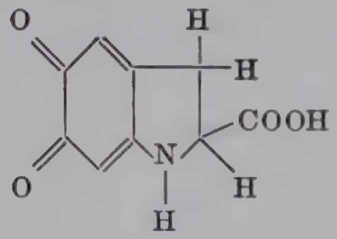
First Phase of Melanogenesis

The theory and assumptions underlying the use of kinetic spectrophotometry have been briefly discussed in another paper (19).

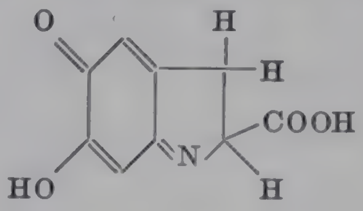
In the first spectrochemical phase of the enzymic transformation of

3,4-dihydroxyphenylalanine into a synthetic melanin, a red pigment with absorption maxima at 305 and 475 mμ accumulates. The rate of formation of this pigment increases with increasing hydroxide ion concentration (Fig. 4) within the limits investigated. The rate of formation has been shown to be dependent upon enzyme concentration ((1), and *cf.* (20)). The rate at which the pigment enters the subsequent reaction in the enzymic sequence leading to melanin also increases with increasing hydroxide ion concentration (Fig. 3, *A*, *B*, and *C*). This observation is in accord with Raper's findings (2) and suggests that at some pH lower than 5.6 (but higher than 2.0; see below) the pigment may be relatively stable. The first spectrochemical phase therefore arises because the red pigment forms more rapidly than it is consumed in the subsequent step. The failure of other intermediates to accumulate has been explained by Evans and Raper ((3) p. 2162).

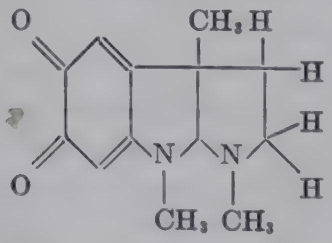
The red pigment may exist in either of two postulated forms, 2-carboxy-2,3-dihydroindole-5,6-quinone (I, *a*) or 2-carboxy-2,3-dihydro-6-hydroxyindole-1,5-quinonimine (I, *b*) (5) (21). Inferences with respect to structure may be made by comparing the absorption spectrum of the pigment with those of substances which may be structurally related. Two such substances are rubreserine (II) and adrenochrome (III), in both of which a fixed 2,3-dihydroindole-5,6-quinone nucleus exists.



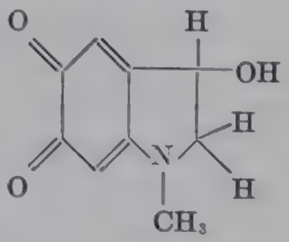
(Ia)



(Ib)



(II)



(III)

The absorption spectra of these compounds, together with those of the red pigment prepared by the silver oxide reaction and by the enzymic oxidation, and finally that of the presumably identical hallachrome (22) are presented in Table II. The positions of the maxima of the red pigment prepared by either procedure and those of rubreserine and adrenochrome are almost identical. The intensities of absorption at these maxima of rubreserine and the red pigment are also almost identical. It is concluded that the observed spectrum of the pigment confirms the nuclear structure already proposed by Raper (5) and establishes the *o*-quinonoid tautomer as that predominating in the pH range 5.6 to 8.4.

Hallachrome is the red pigment isolated from the sea worm *Halla parthenopaea* by Mazza and Stolfi (22) and characterized by them as identical to the red intermediate pigment obtained by enzymic oxidation of 3,4-dihydroxyphenylalanine. However, it is now found that the reported absorption spectrum of hallachrome differs from that of the red pigment (Table I) in that it shows a maximum in the visible region at 539 mμ.

TABLE II

Absorption Spectra of Red Pigment and Related Compounds

Substance	Solvent	Maximum	Log ε	Maximum	Log ε
		mμ		mμ	
Red pigment (silver oxide reaction)	Buffer, pH 5.6	305	3.97*	475	3.54*
Red pigment (enzymic reaction)	" " 5.6	305	3.97*	475	3.57*
Red pigment (enzymic reaction)	" " 6.8 or 8.4	305	†	475	†
Rubreserine (23)	Water	300	3.97‡	480	3.44‡
Adrenochrome (23)	"	305	§	480	§
" (24)	"	310	§	285	§
Hallachrome (22)	Buffer, pH 5.6			539	4.21‖

The figures in parentheses are bibliographic references.

* Calculated on the basis of the structure proposed by Raper (5).

† Decomposition was too rapid at these hydrogen ion concentrations to permit determination of optimal molecular extinction coefficients.

‡ Calculated from data originally published in graphic form.

§ Extinction coefficients not reported.

‖ Recalculated from data expressed in other terms.

Second Phase of Melanogenesis

The second spectrochemical phase of the enzymic transformation of 3,4-dihydroxyphenylalanine into melanin was too elusive to be observed at any but pH 6.8. At this pH level the spectrum of the red pigment disappeared and two new maxima, at 300 and at 540 mμ, developed (Fig. 2, *B*, Curve 1).

If the sequence of reactions taking place in the presence of tyrosinase involves the rearrangement of the red pigment, the second phase spectrum should be identical with either that of the rearrangement product itself or with that of a substance derived from this product. The latter relationship was found to obtain. The rearrangement product, prepared by maintaining the red pigment at pH 5.6 in the absence of oxygen, possessed absorption maxima at 275 and 298 mμ with no absorption in the visible region (Fig. 2, *A*), sharply differing from the second phase spectrum. In similar but not identical experiments Raper (5) has shown that the rearrangement of the red pigment may yield 5,6-dihydroxyindole. In the present experiments the substance formed was identified as 5,6-dihydroxyindole by the similarity of its absorption spectrum to that of 5,6-dimethoxyindole (Table III), there being in general little difference between the positions of the maxima of undissociated phenols and their methyl ethers (25, 26). These spectral curves are, furthermore, unlike those of 2-carboxy-5,6-dimethoxyindole (Fig. 6) and any of a variety of indole derivatives already reported (27–29).

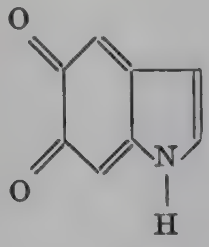
TABLE III

Absorption Spectra of Rearrangement Products of Red Pigment and Related Compounds

Substance	Solvent	Maximum	Log ε	Maximum	Log ε
		mμ		mμ	
5,6-Dimethoxyindole....	Ethanol	272	3.68	296	3.86
Rearrangement product (pH 5.6 reaction)......	Buffer, pH 5.6	275		298	
Rearrangement product (pH 1.3 reaction)......	" " 1.3	310			
Rearrangement product (pH 1.3 reaction)......	" " 5.6	310			
Rearrangement product (pH 1.3 reaction after standing 24 hrs. at pH 5.6)..................	" " 5.6	310			
2-Carboxy-5,6-dimethoxyindole.............	Ethanol	319	4.15		

However, when solutions of 5,6-dihydroxyindole, obtained by the rearrangement of 2-carboxy-2,3-dihydroindole-5,6-quinone at pH 5.6, are subjected to gentle oxidation in the presence of tyrosinase at either pH 5.6 or 6.8, a new absorption spectrum develops. It is characterized by maxima at 300 and 540 mμ (Fig. 2, *B*, Curves 2 and 3). The identity of these spectral curves with that characteristic of the second phase of tyrosinase oxidation of 3,4-dihydroxyphenylalanine (Curve 1) is evident. Differences in intensity are due to loss of substrate during the stepwise preparation of 5,6-dihydroxyindole.

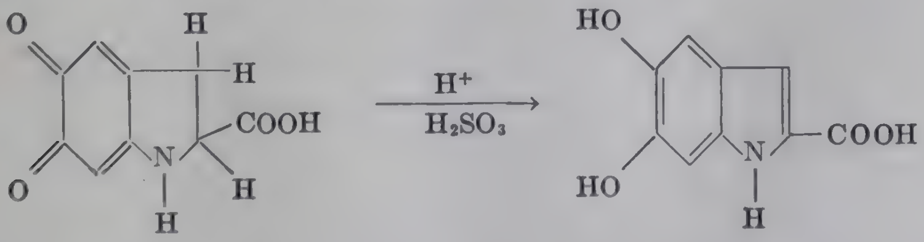
In view of the proved dehydrogenation of catechols to *o*-quinones in the presence of tyrosinase (30) and the catecholic nature of 5,6-dihydroxyindole, it may be inferred that the purple pigment possessing the absorption spectrum with maxima at 300 and 540 mμ is indole-5,6-quinone (IV) or its quinonimine tautomer.



(IV)

The second spectrochemical phase then arises because the purple pigment forms more rapidly than it is consumed in the subsequent step. In view of the instability of 2-carboxy-2,3-dihydroindole-5,6-quinone, these data also suggest that the absorption spectrum of hallachrome (Table II) measured by Mazza and Stolfi (22) was in fact that of indole-5,6-quinone. The chemical evidence in support of the structure of hallachrome which these investigators advanced remains, however, sufficient to establish the identity of hallachrome itself with 2-carboxy-2,3-dihydroindole-5,6-quinone.

When the rearrangement of 2-carboxy-2,3-dihydroindole-5,6-quinone takes place in acid solutions there is formed a product, the spectral curve of which (Fig. 2, *A*, Curve 3) is closely related to that of 2-carboxy-5,6-dimethoxyindole (Fig. 6, Curve 4). The formation of 2-carboxy-5,6-dihydroxyindole as a result of acid rearrangement is therefore indicated. This observation explains why reduction of a 2,3-dihydroindolequinone with sulfurous acid should yield a dihydroxyindole (5, 22):



The present evidence thus indicates that the rearrangement is subject to both hydroxide and hydrogen ion catalysis. The former mechanism is shown to lead to 5,6-dihydroxyindole and the latter to 2-carboxy-5,6-dihydroxyindole.

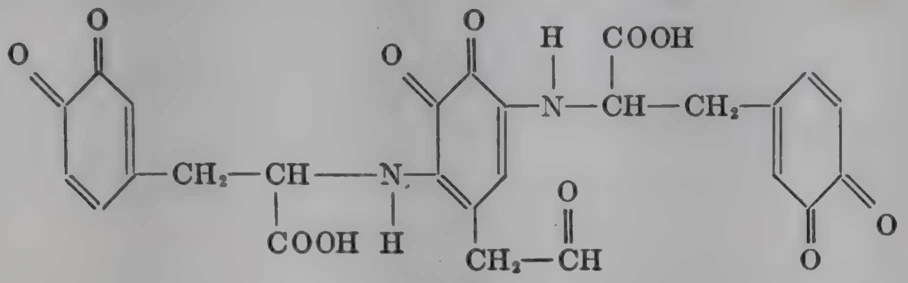
The identity of absorption spectra of 2-carboxy-5,6-dihydroxyindole at pH 1.3 and pH 5.6 (Table III) suggests that this substance does not exist as a cation at either hydrogen ion concentration (26). The stability of its spectrum at pH 5.6 for 24 hours in the absence of oxygen (Table III), a condition under which the red pigment rearranges to 5,6-dihydroxyindole, proves that, during the rearrangement at pH 5.6, 2-carboxy-5,6-dihydroxyindole is not formed first, to undergo subsequent decarboxylation to 5,6-dihydroxyindole.

Third Phase of Melanogenesis

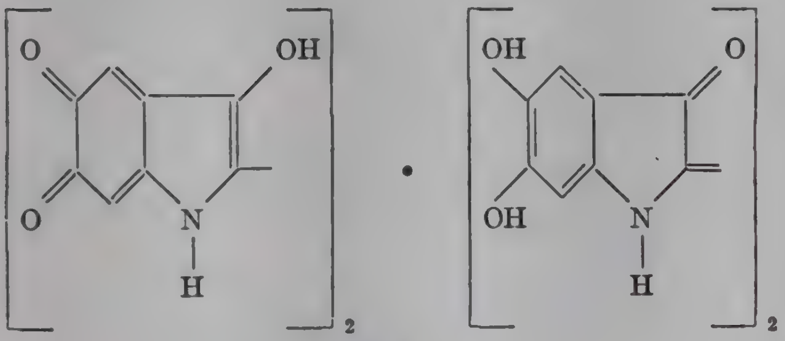
In the third spectrochemical phase of the enzymic formation of a melanin from 3,4-dihydroxyphenylalanine, general absorption became apparent at each of the three hydrogen ion concentrations investigated (Fig. 5). Some specific absorption was also observed in the ultraviolet region. The general absorption may best be interpreted by postulating that the resonating forms of the monomeric melanogen have coupled to produce distributed molecular sizes of a polymer containing one or more long systems of linear oscillators. Some scattering may also be present.

A variety of melanins, both synthetic and derived, have been reported to display only general absorption (12, 14, 31–35). Some positive conclusions have been drawn as a result of the rough similarities between the absorption spectra of these polymeric or high molecular weight materials (14, 34), but in view of the non-specificity of the absorption, the lack of chemical characterizations, and the colloidal nature of the absorbing substances, and, finally, because of the presence of undefined concomitant materials, it does not seem possible to refer similarities or differences in absorption spectra to similarities or differences in the structures of the pigments involved.

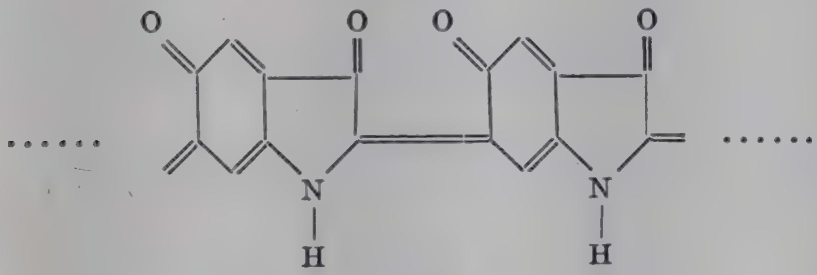
Several hypotheses have been advanced for the structure of the melanin formed by the enzymic oxidation of 3,4-dihydroxyphenylalanine. Bloch and Schaaf (12) suggested that the 3,4-quinone of phenylalanine undergoes intermolecular condensation with simultaneous deamination, leading to (V). This position is no longer tenable in view of the findings of Raper (5). Clemo and Weiss (36) suggest that 5,6,5′,6′-tetrahydroxyindigo is formed from 5,6-dihydroxyindole by a non-enzymic oxidation of the heterocyclic ring, and that this substance, as its quinone or a molecular compound (VI), is an important constituent of melanin pigments. Cohen (37–39) has proposed that 5,6-dihydroxyindoxyl is formed from 5,6-dihydroxyindole and that melanin subsequently appears because of an indoxyl-isatin type condensation (VII). However, it has now been shown that 5,6-dihydroxyindole is readily oxidized in the presence of tyrosinase. Since the most probable product, indole-5,6-quinone, is a molecule possessing the quinone, amine, and pyrrole functions, and since there is considerable evidence establishing the reactivity of the first with the second and third (40, 41), it now seems reasonable to regard indole-5,6-quinone as at least a bifunctional monomer capable of undergoing coupling with itself. The polymer thus arising, or its quinonoid oxidation product, would accordingly be synthetic dihydroxyphenylalalanine melanin.



(V)



(VI)



(VII)

SUMMARY

1. The enzymic oxidation of 3,4-dihydroxyphenylalanine proceeds in three chromophoric phases which are characterized by absorption spectra with maxima at (*a*) 305 and 475 mμ, (*b*) 300 and 540 mμ, and by (*c*) general absorption.

2. The first chromophoric phase was found to correspond to the formation of 2-carboxy-2,3-dihydroindole-5,6-quinone. This tautomeric form of the substance was found to predominate in solution in the pH range investigated, 5.6 to 8.4.

3. 2-Carboxy-2,3-dihydroindole-5,6-quinone rearranges to 5,6-dihy-

droxyindole upon standing at pH 5.6 to 6.8. When 5,6-dihydroxyindole is enzymically oxidized, a product is obtained, the spectrum of which corresponds to that of the second phase of the enzymic oxidation of 3,4-dihydroxyphenylalanine.

4. 2-Carboxy-2,3-dihydroindole-5,6-quinone rearranges to 2-carboxy-5,6-dihydroxyindole upon standing at pH 1.3 to 2.0.

5. The spectral curves of melanins formed by the enzymic oxidation of 3,4-dihydroxyphenylalanine at pH 5.6, 6.8, and 8.4 display general absorption.

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EXCRETION IN REPTILES

II. NITROGEN CONSTITUENTS OF THE URINARY CONCRETIONS OF THE OVIPAROUS SNAKE ZAMENIS DIADEMA, SCHLEGEL

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(Received for publication, September 2, 1947)

Some of the urinary nitrogen constituents have been determined for a few snakes. Boussingault (1) found that 80 per cent of the excretory nitrogen of the boa constrictor and the python is in the form of uric acid. Bacon (2) claims that uric acid in the python amounts to 89 per cent of the total nitrogen, while 8.7 per cent is in the form of ammonia and 2.3 per cent in the form of amino acids, creatine, and creatinine. Girod (3), working on *Tropidonotus natrix*, found that uric acid represents 80 per cent of the excretory nitrogen. The present work was undertaken to secure more detailed analyses of the urinary constituents of snakes.

Material and Methods

Zamenis diadema excretes its urinary constituents in the form of wet masses or concretions. There is no fluid urine. These urinary masses dry up quickly on exposure to the air to form brittle, greenish yellow or white concretions. Microscopical examination of these urinary masses shows that they consist mostly of amorphous granules together with minute globular masses showing striations radiating from the center of each globule. The urinary concretions employed in the present work were collected during a period of 1 month from fifteen animals kept in the laboratory under conditions as similar as possible to their natural habitat. The animals took their food regularly, increased in body weight, and appeared healthy. Urinary concretions were freed under the microscope from fecal and sand particles sticking to them, ground up into a fine powder, and left in the desiccator for 24 hours.

The procedure adopted for the analysis was as follows: Total nitrogen was estimated for a certain weight of the powdered urinary deposit. Another weighed portion was dissolved in warm water, left to cool, and then filtered on a silica filter funnel of known weight. The filtrate was diluted to a definite volume and specimens from this filtrate were analyzed for total nitrogen, urea, ammonia, uric acid (soluble urates), amino acids, creatinine, creatine, and allantoin. The funnel with the water-insoluble portion was left to dry to a constant weight in the desiccator, after which the weight of the insoluble portion was estimated and the total nitrogen determined for a known weight of the insoluble portion. The funnel with the remaining insoluble part was weighed and its contents dissolved in a 1 per cent solution of lithium hydroxide. This latter solution was diluted to a known volume and specimens from it were analyzed for total nitrogen and uric acid. The funnel was left to dry to a constant weight in the desiccator and then weighed. Hippuric acid was also determined on a known weight of the original powdered deposit. Another weighed amount of this deposit was dissolved in dilute sodium carbonate solution and was used for the estimation of the total purine bases by the Krüger and Schmid method as modified by Benedict and Saiki (4). Methods of analysis are the same as those used in Paper I (5). Results are calculated to 1 gm. of the original powdered substance.

TABLE I

Nitrogen Constituents Found in 1 Gm. of Urinary Concretions of Zamenis diadema

Constituents	Nitrogen	N partition in per cent of total N excreted
	mg.	
Total nitrogen	315.3	100
" " in water-soluble portion	98.6	31.27
" " " water-insoluble portion	216.4	68.63
Urea in water-soluble portion	6.4	2.03
Ammonia in water-soluble portion	13.7	4.35
Uric acid " " "	36.8	11.67
Amino acids in water-soluble portion	14.3	4.54
Creatinine " " "	0.1	0.03
Creatine " " "	0.2	0.06
Allantoin " " "	None	None
Uric acid in LiOH solution	174.5	55.34
Hippuric acid in whole deposit	8.5	2.70
Purine bases " " "	6.2	1.97
Undetermined " water-soluble portion	27.1	8.59
Undetermined " water-insoluble portion	27.2	8.6

DISCUSSION

The results of the analysis are shown in Table I.

Although the urinary substances are excreted as solid masses, about 31.27 per cent of the total excretory nitrogenous compounds exists in a water-soluble form. Urea and ammonia form together only 20 per cent of that soluble portion.

Uric acid represents the major nitrogen end-product, but values obtained in the present work for the percentage of uric acid nitrogen to total nitrogen excreted are lower than those obtained by earlier workers. Moreover, about 17 per cent of the uric acid excreted is in the form of soluble urates.

SUMMARY

1. Urinary substances of *Zamenis diadema* are excreted as solid concretions. No fluid urine exists.
2. About 31.3 per cent of the excretory nitrogenous compounds is water-soluble. 20 per cent only of the soluble portion is present as urea and ammonia.
3. Uric acid amounts to 67 per cent of the total excretory nitrogen. About 17 per cent of the uric acid is in the form of soluble urates.
4. Allantoin is absent.

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EXCRETION IN REPTILES

III. NITROGEN CONSTITUENTS OF THE URINARY CONCRETIONS OF THE VIVIPAROUS SNAKE ERYX THEBAICUS, REUSS

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(Received for publication, September 2, 1947)

As far as it could be ascertained, no analysis of the urinary constituents of any viviparous snake has been carried out.

Material and Methods

The urinary constituents of this animal are excreted in the form of dry masses or concretions. They are generally white in color, but they sometimes contain greenish yellow patches. Unlike those of *Zamenis diadema* (1) they are not brittle, but rather hard. When examined microscopically they were found to consist principally of club-shaped crystals together with rosettes and globular crystals showing radiating striations. The material used in the present work was collected from nine healthy animals kept in the laboratory. The procedure followed in the present investigation is the same as that of Paper II (1).

DISCUSSION

The results of the analysis are shown in Table I.

It is significant that although *Eryx thebaicus* is a viviparous animal the main nitrogen end-product is in the form of uric acid; thus the snake does not differ in this respect from oviparous species. This finding does not follow the provisional generalization enunciated by Needham (2) in regard to nitrogen excretion in animals. But discussion of the bearing of this finding on Needham's view should be postponed until the work on nitrogen excretion of the embryos of this animal, as well as that of other viviparous reptiles, is complete.

The percentage of the water-soluble excretory nitrogen constituents is slightly higher than that of the oviparous snake *Zamenis diadema* (1), but about 80 per cent of that soluble portion exists in an as yet unidentified form.

Moreover, all of the uric acid is excreted in the insoluble acid form; no soluble urates are present as is the case with *Zamenis diadema* (1).

While urea constituted about 2 per cent of the total excretory nitrogen in the oviparous snake *Zamenis diadema* (1), it is completely absent from the urinary concretions of this viviparous snake.

TABLE I

Nitrogen Constituents Found in 1 Gm. of Urinary Concretions of Eryx thebaicus

Constituents	Nitrogen	N partition in per cent of total N excreted
	mg.	
Total nitrogen	294	100
" " in water-soluble portion	107.2	36.46
" " " water-insoluble portion	186.2	63.43
Urea in water-soluble portion	None	None
Ammonia in water-soluble portion	16.8	5.71
Uric acid " " "	1.0	0.34
Amino acids in water-soluble portion	3.2	1.09
Creatinine " " "	0.1	0.03
Creatine " " "	0.6	0.2
Allantoin " " "	None	None
Uric acid in LiOH solution	183.8	62.5
Hippuric acid in whole deposit	2.8	0.95
Purine bases " " "	0.9	0.31
Undetermined in water-soluble portion	85.5	29.08

SUMMARY

1. Urinary constituents are excreted in the form of dry solid concretions.

2. Although *Eryx thebaicus* is a viviparous animal, its main excretory nitrogen is in form of uric acid. All the uric acid exists in the insoluble acid form and it amounts to 62.5 per cent of the total excretory nitrogen.

3. About 36.5 per cent of the total excretory nitrogen exists as water-soluble constituents, but 80 per cent of this soluble portion exists in an unidentified form.

4. Urea and allantoin are absent.

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DECREASE OF GLYCINE AND GLUTAMINE IN SKELETAL MUSCLE AND OF GLUTAMINE IN LIVER IN ASCORBIC ACID DEFICIENCY IN THE GUINEA PIG

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(Received for publication, September 23, 1947)

This communication reports an observation of the occurrence, in ascorbic acid deficiency in guinea pigs, of a large decrease of the glycine and glutamine of skeletal muscle and of the glutamine of the liver. While the α-amino nitrogen of glutamine of muscle normally was equivalent to about 25 per cent of the non-glutamine α-amino nitrogen, and glycine nitrogen represented about 35 per cent of the non-glutamine α-amino nitrogen, in the deficiency these values fell to averages of 7 and 13 per cent respectively. The decrease in the glutamine of liver was of similar size. Amino acids other than glycine and glutamine tended to be increased in liver, in plasma, and especially in muscle. Guinea pigs restricted in food intake to obtain weight loss similar to that of deficient animals did not show significant changes in glycine and glutamine distribution.

EXPERIMENTAL

Ascorbic acid deficiency was produced by feeding male guinea pigs (Rockland Farms), weighing 200 to 250 gm., the Rockland rabbit diet. These animals continued to gain for a week, and then, after a day or two, lost weight at the rate of 8 to 10 gm. per day. They were sacrificed and the tissues analyzed between the 13th and 20th days of the régime. An animal sacrificed on the 10th day showed no obvious hemorrhage in the muscles and analysis gave a nearly normal amino acid distribution. The tissues of a 430 gm. guinea pig, showing spontaneous mild scurvy after maintenance for months upon guinea pig ration alone, gave analyses similar to the more acutely deficient animals. This animal was selected by chance for a preliminary trial of the experimental procedure, leading us to the observation reported here.

A first group of controls included ten animals of both sexes weighing 350 to 550 gm. These also served to control other experiments to be reported. They were fed the Rockland guinea pig diet *ad libitum*, with the addition of greens two or three times a week. A second group of three males were random selections from a group of seven uniform animals, the other four being included in the experimental group. These controls were fed the Rockland guinea pig ration, supplemented on alternate days with additional ascorbic acid. A third group of male controls were fed a very low level of the guinea pig ration (reduced from 10 gm. per day to 3 gm. per day in 6 days, then continued for 4 days at 3 gm. per day) fortified with additional ascorbic acid, in an attempt to simulate the weight loss of the scorbutic animals. Although this régime caused the death of half the animals upon about the 10th day after weight losses of about 20 per cent, the rate of weight loss after the 4th day averaged 6 gm. per day, while the scorbutic animals lost about 9 gm. per day.

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After a 12 hour fast, the guinea pigs were anesthetized lightly with ether, and blood was taken under oil by cardiac puncture and transferred to tubes containing heparin. The animals were killed at once by a blow on the head and the liver and musculature of a hind leg were removed quickly, freed of visible adipose tissue, clipped into small pieces, and divided into two portions which were weighed. The major portion of each tissue was quickly extracted with 10 volumes of saturated picric acid in the Waring blendor. The other smaller aliquot was analyzed for water content (1). By providing an operator for each tissue, it was possible to accomplish the suspension in picric acid within 5 minutes of the death of the animal. A delay of an additional 10 minutes did not, however, cause a measurable change in the α-amino nitrogen content found for muscle. Tissues other than liver and muscle have not been examined.

We found that picric acid extracts of plasma or tissues gave findings for chloride indistinguishable from those obtained upon nitric acid extracts (1) or from those obtained after direct ashing. To 15 ml. aliquots of the picric acid extracts were added 1 ml. portions of 0.05 N silver nitrate, ferric alum solution, and 1 ml. of concentrated nitric acid. The resulting solutions, after being concentrated to less than 5 ml. upon a steam bath, were titrated in ice with 0.02 N thiocyanate solution. 0.5 ml. aliquots of plasma were digested directly for chloride analysis. 5 volumes of picric acid were added to the remainder of the plasma (2) to prepare a filtrate low in protein content.

Aliquots of the picric acid extracts of tissues and plasma were treated with neutral lead acetate according to the method of Hamilton (2) to remove glutathione and ascorbic acid. The resulting solutions were analyzed for glycine (3) and glutamine and non-glutamine α-amino nitrogen by the ninhydrin-CO_2 method (2). These filtrates yielded the same results for glycine in plasma as did tungstic acid filtrates, and glycine added to tissues was recovered well in picric acid-lead acetate filtrates.

The extracellular and intracellular water were calculated according to Lowry and Hastings (1) and the intracellular amino acid concentrations calculated under the assumptions that all of the chloride was extracellular, that the extracellular amino acid concentration was 1.05 times that of plasma, and that the additional free amino acids of tissues were in the cells. The extracellular water of the muscle of the deficient animals was as an average 9 per cent higher, the cellular water 17 per cent lower (upon the basis of fresh tissue weight) than that of normal animals, so that determination of the water distribution was particularly desirable in these experiments. The results are summarized in Table I.

TABLE I

Effect of Ascorbic Acid Deficiency upon Distribution of Amino Acids between Cells and Extracellular Fluid

The amino acids are expressed in mg. of α-amino nitrogen. The mean values are given, followed by the standard deviations.

	Liver	Muscle						
		Glutamine		Glycine			Residual*	
	Glutamine concentration of cell water	Concentration in cell water	$\frac{\text{Cell glutamine}}{\text{Cell non-glutamine}}$	Concentration in cell water	Distribution ratio†	$\frac{\text{Cell glycine}}{\text{Cell non-glutamine}}$	Concentration in cell water	Distribution ratio†
	mg. per 100 gm.	*mg. per 100 gm.*		*mg. per 100 gm.*			*mg. per 100 gm.*	
Controls (11),	5.4	5.4	0.26	7.9	8.2	0.35	13.8	5.2
350–550 gm.	±1.5	±1.7	±0.07	±4.5	±2.1	±0.10	±2.9	±1.3
Controls (3),‡	4.9	5.7	0.24	14.3	8.7	0.49	13.6	4.9
290–330 gm.	±1.3	±1.9	±0.08	±2.8	±2.0	±0.05	±4.5	±1.1
Controls (4),§	6.7	4.4	0.17	7.4		0.29	17.3	
restricted intake, 200–220 gm.	±0.6	±0.8	±0.04	±2.5		±0.08	±1.2	
Scorbutic (5),	1.8	2.2	0.066	4.1	4.2	0.13	24	9.6
200–221 gm.	±1.5	±0.4	±0.027	±2.3	±1.2	±0.05	±4	±4.7

* The α-amino nitrogen after glutamine has been destroyed, less the glycine nitrogen.

† Distribution ratio = cell concentration to extracellular concentration.

‡ A group of seven animals was divided at random to make this group and a group including four of the scorbutic animals.

§ Fed a low level of Rockland guinea pig food, with additional ascorbic acid, to simulate the weight loss of the scorbutic group.

DISCUSSION

The observation that glycine contributes a large and highly variable portion of the amino acids of tissues of guinea pigs, and that the glycine levels tend to vary together in liver, plasma, and muscles, will be discussed in another communication.

The known metabolic relationships between *l*-ascorbic acid and amino acids were reviewed by Mitchell (4) in 1943. These include a breakdown in the metabolism of phenylalanine and tyrosine in ascorbic acid deficiency, and, in addition, several *in vitro* chemical reactions between ascorbic acid and amino acids for which physiological counterparts have not been established. The work of Wohlbach and his associates (5–7) showed that ascorbic acid is in some way involved in collagen formation. A restriction of protein synthesis might result from an inability of the scorbutic animal to maintain normal concentration gradients of glycine, and perhaps of glutamine, between plasma and tissue cells. The high glycine content of collagen may render synthesis of this protein particularly sensitive to the low glycine concentration. The "non-essentiality" of glycine of course does not mean that this amino acid is unnecessary for protein synthesis.

That guinea pigs receiving a diet very low in ascorbic acid lose more body weight than paired controls fed isocalorically has been shown by Anderson and Smith (8) and McHenry, Reedman, and Sheppard (9), although Sheppard and McHenry (10) concluded that the difference was largely due to a decreased retention of water by the deficient animals. McKee, Cobbey, and Geiman (11) recently observed a biochemical defect in gluconeogenesis and glycogenolysis in scorbutic guinea pigs which apparently is a result of an adrenal cortical deficiency. Possibly the defect recorded here may have a similar origin. However, adrenalectomy in the rat apparently increases rather than decreases the concentration gradients of the (total) free amino acids between muscle and plasma, a fall occurring in the concentration in the plasma and a rise in the muscle (12). The excess of protein catabolism in adrenal cortical hyperactivity is well known.

SUMMARY

Scorbutic guinea pigs showed a reduction in the free glycine and glutamine content of muscle cells, and of the glutamine of liver cells much below that seen in normal animals or in fasted controls. Amino acids other than glycine and glutamine were increased in the deficient animals.

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THE ISOLEUCINE CONTENT OF SEED GLOBULINS AND β-LACTOGLOBULIN

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In a recent study of the amino acid composition of some crystalline seed globulins and β-lactoglobulin, the isoleucine content of these proteins was determined by microbiological assay (1). It has now been learned that the commercial sample of DL-isoleucine (Merck) which was used as a standard in these assays contained some DL-alloisoleucine.[1] Since the latter compound apparently cannot replace isoleucine in supporting the growth of *Streptococcus faecalis* which was used in our assays, the values obtained earlier are obviously too high, and new assays have been performed with a sample of DL-isoleucine (Merck) which is known to be free from the allo compound.

The microbiological assays were performed exactly as described earlier and on the same preparations of the crystalline proteins (1). When the two samples of isoleucine used in the previous studies were assayed against the new standard, average isoleucine values of 76 and 88 per cent were found. It is, therefore, not possible to make a blanket correction of the old assays. The new determinations of the isoleucine content of the proteins are given in Table I, together with the values obtained previously.

Less variability in the results was encountered in these measurements than was found earlier; this may be simply a reflection of the use of a single standard. Edestin and β-lactoglobulin gave values which were 76 and 70 per cent of those previously reported. The isoleucine content of the other proteins has now been determined to be about 85.5 per cent (average of 82, 87, 84, and 89 per cent) of the earlier estimations. Thus, the new values are in reasonable accord with the direct assays of the isoleucine content of the old standards.

Our result of 6.1 per cent for the isoleucine content of β-lactoglobulin is in excellent agreement with the 5.86 per cent obtained by Moore and Stein (2) using the starch chromatogram method. These results are lower than the 8.4 per cent reported by Brand *et al.* (3), and the 7.0 per cent found by Stokes *et al.* (4).

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[1] We are indebted to Mr. F. A. Bacher of Merck and Company, Inc., for this information, and for the sample of highly purified DL-isoleucine which was used as a standard in this investigation. According to Mr. Bacher, the purity of the purified isoleucine was greater than 99 per cent, as indicated by solubility studies.

It is probable that some of the studies of the isoleucine content of proteins and other biological materials which have been performed by microbiological assay were made with standards containing some alloisoleucine. Such results would give high values. Unfortunately, optically active isoleucine from proteins is not readily available, and there are no simple satisfactory tests for estimating the DL-alloisoleucine content of any particular sample of DL-isoleucine. Repeated recrystallization from water-ethanol mixtures is reported to separate isoleucine from the allo compound (5).

TABLE I

Isoleucine Content of Seed Globulins and β-Lactoglobulin

The values are averages for four separate hydrolyses and are given for the anhydrous ash-free proteins. For each hydrolysate, the average of several measurements at different growth levels is regarded as a single determination.

Protein	Isoleucine content	Old isoleucine value, Smith and Greene (1)
	per cent	*per cent*
Edestin	4.7 ± 0.1	6.2
Pumpkin	4.6 ± 0.2	5.3
Watermelon	4.7 ± 0.1	5.7
Cucumber	4.9 ± 0.1	5.5
Tobacco	5.3 ± 0.1	6.3
β-Lactoglobulin	6.1 ± 0.1	8.7

SUMMARY

New analyses are reported for the isoleucine content of several crystalline seed globulins and β-lactoglobulin, as determined by microbiological assay with *Streptococcus faecalis*. Earlier results were found to be too high because the standards of DL-isoleucine which were used contained some DL-alloisoleucine which is inactive biologically.

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CHANGES IN RAT LIVER ENZYME ACTIVITY WITH ACUTE INANITION*

RELATION OF LOSS OF ENZYME ACTIVITY TO LIVER PROTEIN LOSS

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The exact mechanism of the increased susceptibility to poisons of the liver in the protein-depleted dog, rat, and human remains obscure. A previous report (1) raised the question of a general loss of activity of the liver enzymes as being the result of protein depletion. But without a knowledge of the precise mode of action of such toxic agents as chloroform, it is not possible to focus a study on the immediate problem. However, the effect of protein depletion on enzyme activity in general seems fundamentally important enough to warrant a study of the effect of inanition and the resulting liver protein depletion on a number of liver enzymes.

Addis *et al.* (2) have systematically studied the tissue protein lost by the rat during fasting for periods up to 7 days, in which time the liver may lose 40 per cent of its protein. Kosterlitz (3) has extended the study to include and correlate changes in liver phospholipide and nucleoprotein. Kaplansky *et al.* (4) have described the decrease of deaminating, transaminating, and glycogen-forming functions in protein-depleted rats.

This report presents data on changes in activity in rat liver enzyme with acute inanition induced by a 7 day fast. The enzymes studied were selected because they represent enzymes of different functional classes, because their intracellular distributions differ, and because their activity may be measured in multiple fashion without manometric techniques. The observed changes in catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin activity parallel or exceed the loss of liver protein. It thus appears that the biochemically functional proteins of the liver cell (*i.e.*, the enzymes) are sacrificed along with cell proteins in general. These findings relate to a number of important questions concerning (1) the metabolic relation of enzymes to recognized morphologic structures of the liver cell, (2) the relative stability of intracellular enzyme proteins under conditions of organ protein catabolism or loss, (3) the importance of dietary protein in maintaining not only the integrity of organ protein in general but enzyme proteins in particular, and (4) the relation of dietary protein to the phenomenon of enzyme adaptation in higher mammals.

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* Part of this report was presented at the meeting of the American Society of Biological Chemists at Chicago, May 22, 1947 (*Federation Proc.*, **6**, 279 (1947)).

Methods

Male and female rats of Sherman strain, weighing 225 to 350 gm. and 175 to 250 gm. respectively, were maintained on a stock synthetic diet for at least 10 days prior to their use. The diet contained vitamin-free casein (General Biochemicals, Inc.) 25 per cent, glucose, anhydrous Merck, 61 per cent, Mazola oil 10 per cent, salt mixture U. S. P. XII (General Biochemicals, Inc.) 4 per cent, and the following vitamins[1] per kilo: thiamine 10 mg., riboflavin 10 mg., pyridoxine 10 mg., calcium pantothenate 30 mg., niacin 25 mg., choline chloride 1 gm., menadione 4 mg., inositol 500 mg., vitamin A 60,000 I.U., and vitamin D 1500 I.U. Control animals were allowed food until immediately before sacrifice. Fasted rats were placed in individual cages and allowed only water *ad libitum* for 7 days. The rats were killed by stunning, transection of the great vessels of the neck, and exsanguination. The livers were rapidly removed *in toto*, blotted free of blood with filter paper, and homogenized with a volume of double distilled water equal to 9 times the weight of the tissue used. 3 minutes homogenization in the Waring blendor gave stable suspensions in which neither intact cells nor nuclei could be detected after staining with methylene blue.

Catalase activity was measured by diluting the original homogenate to 100 times its volume with 0.02 M phosphate buffer, pH 6.8, and using 1 ml. aliquots of the resulting 1:1000 suspension according to the method of von Euler and Josephson as modified by Jolles (5). The units of activity per gm. of liver were obtained by multiplying the unimolecular reaction constant (extrapolated to zero time) by 51,000.

Alkaline phosphatase activity was measured with the 1:10 homogenate by the method of Bodansky (6) at 37°, pH 9.2 to 9.3, and the phosphate liberated was estimated by the method of Fiske and Subbarow (7). The units of phosphatase activity are expressed in terms of mg. of P liberated per gm. of liver per hour.

Xanthine dehydrogenase activity was measured at 37° and pH 7.6 according to the Thunberg technique described by Figge and Strong (8), except that the homogenate was dialyzed against several changes of distilled water in the refrigerator for 24 hours. Units of activity are based on the definition of Wieland (9), 1 unit arbitrarily decolorizing 1 ml. of 0.001 M methylene blue in 5 minutes at 37°.

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[1] We are indebted to Merck and Company, Inc., Rahway, New Jersey, for a generous supply of vitamins.

Cathepsin activity was measured at 37° and pH 3.7 to 3.8 by mixing a 1 ml. aliquot of the 1:10 homogenate with 5 ml. of dog hemoglobin denatured by the method of Anson (10). After exactly 0 and 3 hours, the reaction was stopped by mixing the contents with 10 ml. of 5 per cent trichloroacetic acid. The resulting suspension was centrifuged and 4 ml. aliquots of the supernatant were analyzed in triplicate for N by the micro-Kjeldahl procedure. The increase in soluble N as a result of cathepsin activity is expressed in terms of mg. of hemoglobin N made soluble per gm. of liver in 3 hours.

The nitrogen content of the 1:10 homogenate was determined by the micro-Kjeldahl procedure in triplicate on 0.5 ml. aliquots, and the N content thus determined is assumed as a measure of the protein content (*i.e.*, per cent liver protein = per cent N × 6.25).

The activity of all the enzymes studied is also expressed in terms of units per 100 gm. of body weight and in terms of units per gm. of liver protein. Both these methods of expression eliminate variations seen in the figures for "units per gm. of liver" as a result of dilution by glycogen or lipide.

Expressing results in units per 100 gm. of body weight has been justified as being a method of comparing total activity in animals of different weight, and also is representative of the total enzyme activity for a given animal. In the fasted animals, the "initial body weight" is used for this computation because it is comparable to the initial weight of the liver, from which losses have occurred.

The enzyme activities in terms of units per gm. of liver protein allow a direct comparison of the loss of enzyme activity with the loss of liver protein in general, and immediately reveal whether enzyme protein (activity) is preferentially conserved, lost at the same rate, or lost in excess of organ protein in general.

Results

Table I presents a comparison of liver catalase activity in normal fed rats with fasted rats. In the case of liver catalase, the previously reported difference (11) between males and females is confirmed, and the results obtained from the males and females are tabulated separately. It is clearly apparent that the loss of catalase activity is more pronounced in the females than in the males. In the former the loss exceeds the general protein loss; so that the activity in units per gm. of liver protein after fasting is significantly less than in the fed females. In the males the loss of catalase activity parallels the protein loss with no significant change in activity per gm. of liver protein. The loss of catalase activity in both sexes is more pronounced when it is expressed in terms of units per 100 gm. of initial body weight.

It is also noteworthy to compare the catalase activity after fasting with the activity calculated on the assumptions that the enzyme protein is completely spared and that the enzyme protein represents an insignificantly small fraction of the total protein. This indicates that there has certainly been no preferential conservation of enzyme protein.

TABLE I

Liver Catalase Activity

	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight*	Units per gm. liver protein*	Calculated,† units per gm. liver protein
Females, fed	10	1740	5,980 ± 1940	8.730 ± 2900	
" fasted	7	1210	2,540 ± 930	5,450 ± 1800	12,540
Males, fed	10	2950	10.580 ± 2010	14.580 ± 2140	
" fasted	6	2860	6,070 ± 2060	12,620 ± 3500	22,000

* The data are given with calculated standard deviations from the mean.

† Calculation of expected mean activity on the assumptions that none of initial enzyme activity is lost, and that enzyme protein is an insignificantly small part of total protein. Calculation based on average liver protein of 668 and 713 mg. per 100 gm. of initial body weight in fed females and males, and 465 and 472 mg. per 100 gm. of initial body weight in fasted females and males respectively.

TABLE II

Liver Alkaline Phosphatase Activity

	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight*	Units per gm. liver protein*	Calculated,* units per gm. liver protein
Females, fed	10	1.18	4.10 ± 1.12	5.92 ± 1.80	
" fasted	6	1.29	2.73 ± 0.57	5.66 ± 0.81	8.50
Males, fed	10	1.23	4.47 ± 0.47	6.10 ± 0.77	
" fasted	6	1.46	2.54 ± 0.83	6.44 ± 1.07	9.21

* See foot-notes to Table I.

Table II presents a comparison of liver alkaline phosphatase activity in 7 day-fasted and fed animals. The results for both sexes are separated, but the difference between the sexes is not significant. Here the alkaline phosphatase activity is lost at about the same rate as the liver protein; so that the activity in units per gm. of liver protein remains relatively constant after the 7 day fast. The loss in total activity becomes apparent when it is expressed in units per 100 gm. of initial body weight. This loss becomes amplified when the activity found in units per gm. of liver protein is compared with the activity calculated on the assumption that the enzyme protein (and activity) is spared, while the protein in general is lost.

Since much of the liver alkaline phosphatase activity is associated with the particulate matter ("large granules" of Claude) of the liver cytoplasm, and since the number of these particles has long been known to decrease with fasting, the fate of the enzymic components of these particles appears to be the same; *viz.*, they are lost to the cell along with other proteins, phospholipide, and ribonucleic acid.

Table III shows the striking effect of a 7 day fast on the liver xanthine dehydrogenase activity of both male and female rats, the changes being essentially the same for both sexes. Of the enzymes studied, the xanthine dehydrogenase shows the greatest loss in activity, the loss being greatly amplified when the observed activity per gm. of liver protein is compared with the calculated activity, assuming that the enzyme protein is retained and represents an insignificant portion of the total protein.

TABLE III

Liver Xanthine Dehydrogenase Activity

	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight*	Units per gm. liver protein*	Calculated,* units per gm. liver protein
Females, fed	10	2.0	7.0 ± 2.4	10.2 ± 3.3	
" fasted	7	1.1	2.3 ± 1.2	4.7 ± 1.7	14.7
Males, fed	10	2.4	8.6 ± 2.5	12.0 ± 3.0	
" fasted	6	1.1	2.2 ± 1.1	4.9 ± 1.6	18.1

* See foot-notes to Table I.

As Figge and Strong have pointed out (8), we have found it essential to dialyze the homogenates to eliminate low molecular weight metabolites, which with other liver enzymes may rapidly reduce methylene blue without added xanthine. The 24 hour dialysis in the refrigerator against several changes of distilled water removes most of the extraneous metabolites, since the methylene blue in numerous blanks was not completely reduced in over 3 hours time, while the addition of xanthine resulted in complete reduction of the methylene blue in 20 to 40 minutes with most of the livers from fed animals.

Table IV shows changes in liver cathepsin activity in animals fasted 7 days compared with fed animals. The loss of activity appears to lag behind the protein loss as manifest by a small but significant increase in the activity in units per gm. of liver protein. This apparent increase is considerably less than the activity calculated on the assumptions that no enzyme protein (activity) is lost and that the enzyme protein represents a very small fraction of the total. Although it appears that the average liver cathepsin activity of females is higher than that of males, statistically there is about 1 chance in 4 that they may be the same.

TABLE IV

Liver Cathepsin Activity

	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight*	Units per gm. liver protein*	Calculated,* units per gm. liver protein
Females, fed	1	21.0	72.1 ± 28.6	106 ± 33.9	
“ fasted	7	24.7	48.6 ± 10.5	110 ± 25.3	152
Males, fed	10	15.9	60.2 ± 9.3	81.4 ± 12.7	
“ fasted	6	21.0	43.2 ± 6.3	92.0 ± 12.4	123

* See foot-notes to Table I.

DISCUSSION

Before the significance of the above data is discussed, it is necessary to examine the validity of the assumption that enzyme activity as measured is proportional to enzyme protein under the conditions of these experiments and that any decrease of activity observed is a result of a net loss of enzyme protein *per se*, and not a result of a defect in prosthetic groups, or of the presence of an inhibitor, or loss of an activator.

Liver catalase is an iron porphyrin protein complex and its activity is known to be reduced by iron or Mn deficiencies (11) over lengthy periods of time, presumably as a result of failure of production of the iron porphyrin complex. In our experiments the animals are adults and time is too short for a mineral deficiency; in addition it is known that iron porphyrin production for the related pigment hemoglobin can proceed under conditions of maximal stimulus for hemoglobin production in fasted dogs. Xanthine dehydrogenase is a flavoprotein, and it is well recognized that prolonged dietary restriction of certain vitamins of the B complex (*e.g.* riboflavin or thiamine) may result in a marked decrease in the enzyme (*i.e.* activity) which contains these vitamins as an integral portion of the prosthetic group or enzyme complex. Here again the time elapsed is too short for a vitamin deficiency; in fact measurements of changes in liver vitamins with 4 days partial fasting and weight loss reveal an increased concentration of liver riboflavin (12). It therefore seems more likely that the loss of xanthine dehydrogenase activity is referable to the loss of the protein moiety of this enzyme. Evidence for a decrease in the protein moiety during prolonged riboflavin deficiency has been adduced by Axelrod and Elvehjem (13).

To determine whether the 7 day fast causes the accumulation of inhibitors or loss of activators of enzyme activity, homogenates of normal liver and of liver from fasted rats were examined for catalase, xanthine dehydrogenase, and cathepsin activity, singly, and in suitable admixtures. The resulting activity was equal, within experimental error, to the activity calculated from the contribution of each component, making the presence of an inhibitor or loss of an activator unlikely.

If the parallelism between enzyme activity and enzyme protein is here acceptable, it at once becomes apparent that vital though the enzymes may be regarded their protein is readily lost from the cell. This loss occurs at a rate which varies with the different enzymes, the loss of cathepsin being somewhat less than the general protein loss, that of catalase and alkaline phosphatase roughly paralleling or exceeding the protein loss, and that of xanthine dehydrogenase greatly exceeding the general protein loss.

Since the activity of all four enzymes, each of fundamentally different functional type, decreases with the loss of cell protein, it appears highly probable that the activity of other enzymes, if measured, would be found similarly reduced. Potter and Klug (14) have reported decreased octanoate oxidase and succinic dehydrogenase activity in homogenates of livers of rats maintained on diets containing 83 per cent fat and 87 per cent carbohydrate, respectively, and only 6 to 10 per cent protein. Neither food consumption nor weight loss is described. It is conceivable that the decreased enzyme activity was coincident with a loss of liver protein, including enzyme proteins, as a result of protein depletion. The failure to demonstrate increased (or even normal) activity of these enzymes as an adaptation to the high fat and carbohydrate content of the diets is probably referable to the protein-deficient diet, and, in the case of the high fat diet, to dilution of the liver enzyme by fat (results expressed in units per mg. of wet weight).

Similarly Lightbody and Kleinman (15) have reported that arginase activity of liver homogenates from rats on a 6 per cent milk protein diet is roughly one-half that of the activity from rats on a 25 per cent milk protein diet, and interpret this difference as an adaptation by the liver to the increased need for urea formation on the high protein diet. This conclusion was reached in spite of data showing significantly lower liver arginase activity in the livers of rats on a diet containing 6 per cent milk protein and 19 per cent gelatin. Because of this, and because of experiments now in progress demonstrating decreased liver arginase activity in livers of rats on a diet containing 6 per cent casein and 19 per cent glycine, it appears more likely that arginase (activity) may be reduced below normal along with other liver protein as a result of inadequate protein intake. It appears that when a diet contains an abnormally large amount of a metabolite the enzyme or enzymes involved in its disposal cannot increase in amount unless the diet contains protein adequate for the formation of new liver protein including these enzymes.

The literature on experimental carcinogenesis contains numerous references to decreased enzyme activities in livers of animals with or without tumors (16). In many instances no reference is made to weight loss or to chronic protein depletion which might be anticipated from feeding unpalatable diets very low in protein. In these instances, reduction of enzyme activity and its alleged significance for the origin of, or relation to, the neoplastic changes observed must be reexamined, since similar changes may be found to be the result of simple inanition.

The fact that the decrease in liver size with inanition is associated with a loss of cell cytoplasm and a decrease in the number of cytoplasmic granules suggests the possibility that the enzymes found in the cytoplasm (*e.g.* catalase) or in the large granules (*e.g.* alkaline phosphatase, succinic dehydrogenase, cytochrome oxidase) are also decreased in amount. The above data firmly support this idea and lead to the hypothetical conclusion that with uncompensated liver cell protein catabolism enzyme proteins of all types may be rapidly lost. Whether this loss is the result of increased destruction, reduced formation, or both is not clear. If the cytoplasmic nucleoprotein (the "plasmagene" of Spiegelman and Kamen (17)) is involved in the intracellular synthesis of enzymes, and if this nucleoprotein is decreased in concentration or total amount, then the capacity for enzyme synthesis may be reduced. The conclusion that cell size, the number of mitochondria, cell protein in general, phospholipide, and ribonucleoprotein may decrease in roughly parallel fashion is of great interest, especially when taken together with our observations on the loss of enzyme activity. They support a concept of intracellular functional units made up of a variety of enzymes and associated nucleoprotein and lipoprotein. This concept further implies that the loss of any integral portion of the unit is associated with the loss of the unit as a whole.

Undoubtedly a measurably great excess of enzyme activity is reflected in the great functional reserve of the liver, and the loss of function is clinically measurable only when enzyme activity falls to very low levels. In practice this has long been recognized for such functions as urea and fibrinogen formation.

SUMMARY

1. A 7 day fast results in a loss of rat liver catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin activity, which parallels or exceeds the loss of liver protein.

2. This loss of enzyme activity cannot be explained by a loss of prosthetic groups or enzyme activators or by an accumulation of inhibitors, and probably represents a loss of enzyme protein *per se*.

3. These findings bear on the relation of liver enzymes to morphologic structures, the metabolism of enzyme proteins during liver protein catabolic loss, and on the phenomenon of enzyme adaptation in higher mammals.

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THE BIOCHEMICAL DETERMINATION OF THIOURACIL IN THE MUSCULAR TISSUE OF HOGS RECEIVING SUPPLEMENTARY THIOURACIL IN THE DIET

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Within the past few years the rôle of certain substances, goitrogens, which depress the normal function of the thyroid gland in the rat and other animals has been elucidated. Kennedy in 1942 (1), Astwood and coworkers (2), and Mackenzie and Mackenzie in 1943 (3) demonstrated the effects of goitrogens, specifically certain sulfonamides and a number of substances related to thiourea, on the thyroid gland of the rat. They found that these compounds resulted in hypertrophy and hyperplasia of the thyroid gland coincident with a state of hypothyroidism as indicated by decreased food intake, decreased growth and development, and lowered basal metabolic rate. Thiouracil was found to be the most active compound in this respect (4) and to be the least toxic at concentrations eliciting comparable effects to other goitrogens. Investigations by Reineke *et al.* (5) on the rate of secretion of thyroxine by the rat demonstrated that with administration of thyroxine to thiouracil-treated animals the basal metabolic rate returned to normal.

The rate of thyroxine secretion has been linked by these and other workers with the regulation of the rate of body growth and fattening. Following this assumption Kempster and Turner (6) fed thiouracil at a level of 0.2 per cent in the diet to chicks and noted a marked improvement in carcass grade after a 36 day period. Andrews and Schnetzler (7) corroborated these findings and, in addition, found increased fat deposition and a somewhat greater efficiency of feed utilization over control animals. Glazener and Jull (8) noted that 0.2 per cent thiouracil in the ration of broilers for 8 weeks depressed the growth rate and feed consumption and at the same time their dressed appearance was rated less desirable than that of controls. Mixner *et al.* (9) found a reduction of 25 per cent in feed consumed per pound of gain by young cockerels fed 0.1 per cent thiouracil as compared with control animals for a 4 week period.

Work by Reineke and coworkers (10) showed little or no improvement in the carcass grade of turkeys fed low levels of thiouracil for 3 to 4 weeks. It was found, however, that the feeding efficiency, as indicated by the amount of feed required per unit of weight gain, was increased from 17 to 33 per cent when thiouracil was added to the ration.

Experiments have also been reported on the effects of feeding thiouracil to steers, sheep, and swine. Beeson *et al.* (11) reported a tendency for steers receiving 2 or 4 gm. of thiouracil daily to require less feed per pound of gain and to show an increased rate of growth. A similar study was conducted by Heinemann and coworkers.[1] Two groups of ten young heifers were used. The animals received a grain ration together with a small supplement of hay and one group received thiouracil added to the grain ration at a level of 0.2 per cent. After 100 days it was found that the rate of gain of both groups was about the same, but the group receiving thiouracil required only 5.44 pounds of grain per pound of gain as compared with 6.32 pounds for the control group. The increase in efficiency of food utilization was 14 per cent. However, the control group consumed somewhat more hay than that receiving thiouracil.

In the case of sheep, Andrews *et al.* (12) reported no changes in the rate of gain or feeding efficiency, but a definite improvement in carcass quality when 0.17 or 0.33 gm. of thiouracil was ingested daily. Muhrer and Hogan (13) fed swine a ration containing 0.2 per cent of added thiouracil. After 28 days the experimental animals were shorter and fatter and had gained weight more rapidly and economically than had the controls. The average daily gain of the treated animals was 1.5 times that of the controls, and the increase in feeding efficiency, calculated as pounds of feed consumed per 100 pounds gained, was 36 per cent in the case of the thiouracil-treated animals. Van der Noot *et al.* (14) confirmed the general findings of Muhrer and Hogan. They found that thiouracil-fed hogs made an average daily gain of 1.60 pounds, whereas control hogs gained 1.23 pounds per day. They showed further that hogs fed a ration containing 0.25 per cent thiouracil consumed 27.5 per cent less feed per 100 pounds of gain in body weight than did hogs fed a thiouracil-free ration. Similar results in feeding economy and gain in swine have recently been published by McMillen *et al.* (15). Chester White barrows fed thiouracil at a 0.1 per cent level in the ration for 41 days made 0.16 pound less daily gain than the controls, but required 13.8 per cent less feed per unit of gain. The only reference to carcass grade of thiouracil-fed swine in the literature appears in this report of McMillen *et al.* (15) who found no significant differences attributable to thiouracil treatment.

The recent impetus given to investigations on the feeding efficiency and fattening of farm animals through the use of thiouracil has led to the need for a method for the determination of the compound in meat. The present investigation was undertaken in an attempt to develop such a method.

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[1] Heinemann, W. W., Singleton, H. P., Kline, E. A., Ensminger, M. E., and Cunha, T. J., private communication.

Several methods have been described for the determination of thiouracil in tissue. Williams *et al.* (16) used a modification of the procedure of Nicholes and Herrin (17). The method is based on the development of a blue complex when Grote's reagent is added to an alkaline protein-free filtrate of homogenized tissue. This general method, however, has been subjected to several modifications, chiefly because of the difficulty in obtaining reproducible results in the use of the reagent (18). Turner and associates[2] have found that the development of a pink color upon addition of the reagent to the muscular tissue filtrate interferes in such a way that the method becomes unsatisfactory. Other workers have confirmed this observation and have found considerable difficulty in the use of Grote's reagent for the determination of thiouracil in muscular tissue.[3] The method, however, gives satisfactory results for the measurement of thiouracil in plasma and urine (18).

Pipes and Turner (19) employed a biological method for the determination of thiouracil in chicken tissue. Fresh tissue containing thiouracil was fed to rats for a period of 2 weeks, at which time the thyroids were excised and the hypertrophy, as measured by the thyroid gland weight, was compared with a standard reference curve of gland weights of rats fed increasing amounts of thiouracil added to chicken tissue. This method is not readily applicable to measurement of the thiouracil content of the tissues of animals which have been fed low levels of thiouracil, because the sensitivity of the assay is low and only small amounts of thiouracil are found in such tissues.

The recent work of Chaikoff and associates (20, 21) on the effect of thiouracil on the uptake of injected radioiodine by the thyroid gland and its conversion to thyroxine and diiodotyrosine is the basis of the method described in the present investigation. In studying the radioiodine-concentrating capacity of the thyroids of rats fed thiouracil, they found that a daily feeding of 4 mg. of thiouracil for a week reduced the uptake of administered radioiodine by the rat thyroid so that the glands in the thiouracil-treated animals accumulated only about one-third as much radioiodine as those in the control rats. A corresponding depression was also found in the conversion of iodide to thyroxine and diiodotyrosine as represented (22) by organically bound iodide. Thus, thyroid activity may be measured by the avidity of the gland for iodine or by its ability to synthesize thyroxine and diiodotyrosine. In the present study the degree of inhibition of thyroid activity was found to be a function of the level of dietary thiouracil. This finding was utilized in the development of a method for the determination of small amounts of thiouracil in tissue.

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[2] Turner, C. W., and Pipes, G. W., private communication.

[3] Boehne, J. W., personal observation.

EXPERIMENTAL

Care of Hogs and Preparation of Dried Hog Tissue—Six Chester White weanling hogs weighing approximately 10 kilos served as experimental animals. They were kept in small straw-floored pens in a large barn. Food and water were fed *ad libitum*. The animals were fed a basal ration of Cooperative G. L. F. Mills hog feed having the following open formula: wheat standard middlings, 19 per cent; yellow corn-meal, 46.5 per cent; finely ground, low fiber oats, 10 per cent; alfalfa meal, low fiber, 5 per cent; 41 per cent protein soy bean oil meal, 14 per cent; meat scrap, 55 per cent protein, 3.5 per cent; ground limestone, 1.5 per cent; and iodized salt, 0.5 per cent.

The animals were divided into two groups. Two hogs were used as control animals and received the basal diet throughout the experimental period and provided the control hog tissue used in the preparation of the rat diets.

The other four hogs were fed a supplement of 0.2 per cent thiouracil added to the same basal diet for 34 days. The thiouracil supplementation was then terminated. Two animals were sacrificed and the tissues were treated as described below. The combined dried muscular tissue from these animals is designated as Tissue A in Table I. The two remaining hogs were placed on the basal ration. One was sacrificed 1 day later (Tissue B), and the remaining hog was killed after 3 days on the basal ration (Tissue C).

The animals were bled to death and the muscular tissue was excised. Superficial fat was removed as completely as possible. No internal organs or visceral tissue was collected. The fresh tissue was immediately ground, then spread evenly on galvanized metal trays which were placed in a large circulating hot air oven at 60° for 24 hours. The dried tissue was then reground and stored at 4°.

Care and Treatment of Assay Rats—Weanling female albino rats of the Wistar strain served as experimental animals in the assay. They were housed in wire bottom cages and were fed the basal ration with supplements for 19 days as indicated in Table I and fresh water *ad libitum*. Three rats were used in each group. The use of chick mash as a diluent effected an even distribution of the supplement. On the 18th day all animals were injected intraperitoneally with 1 ml. of an isotonic saline solution containing a tracer dose of 0.1 microcurie of radioiodine as I^{131}. The animals were immediately placed by groups in metabolism cages constructed so that the urine was collected free of feces over the ensuing 24 hour period.

Determination of Distribution of Radioiodine—The rats were sacrificed 24 hours after the administration of the radioactive iodine and the thyroid glands were excised. The glands were weighed and hydrolyzed by autoclaving for 1 hour in 6 ml. of 0.2 N NaOH. The hydrolysates were made up to a volume of 10 ml. For the determination of the total iodine radio-

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activity, an aliquot of the hydrolysate was dried in a porcelain dish on a hot-plate and the radioactivity was measured with a thin mica window Geiger-Müller counting tube. The total labeled iodine obtained from the glandular tissue was expressed as a percentage of the administered dose of radioiodine (Table II). A second aliquot of the hydrolysates was used for the separation of organically bound radioiodine as described by Schachner *et al.* (22).

TABLE I

Diets and Average Weight Changes of Rats Used in Assay for Thiouracil

Group No.	Amount and type of dried muscle present per 100 gm. diet*	Thiouracil added to diet	Feed consumed	Weight gain over experimental period	Weight at end of experimental period
	gm.	*mg. per 100 gm.*	*gm. per day per rat*	*gm.*	*gm.*
1	50 (N)	0	7.5	54	100
2	50 "	0	7.5	47	89
3	50 "	0.1	6.7	51	93
4	50 "	0.5	7.4	46	92
5	50 "	1.0	6.4	43	89
6	50 "	5.0	6.6	39	87
7	50 "	10	5.8	44	83
8	50 "	20	6.3	39	82
9	50 "	50	6.7	46	91
10	50 "	100	6.6	41	81
11	50 (A)	0	6.8	48	97
12	40 (N), 10 (A)	0	6.3	35	81
13	50 (B)	0	7.0	35	80
14	40 (N), 10 (B)	0	6.4	46	90
15	50 (C)	0	6.6	47	91

* All diets contained 50 gm. of chick starter mash (Pratt's) per 100 gm. The types of dried muscle were as follows: N, from hogs on thiouracil-free control diet; A, from hogs killed on day of termination of supplementation of feed with thiouracil; B, from hogs killed 1 day after termination of supplementation; C, from hogs killed 3 days after termination of supplementation. The addition of thiouracil to the diets of Groups 1 to 9 was made together with glucose (cerelose) to a total of 100 mg. per 100 gm. of diet.

Urine samples, representing the total urine excretion of each group of rats immediately following the radioiodine injection over the last 24 hours of the experimental period, were collected and suitable aliquots were removed for separation of organically bound radioiodine as well as for the determination of total iodine radioactivity. The labeled iodine found was expressed as a percentage of the administered dose of radioiodine (Table II).

Results

A number of biochemical measurements were made during the course of the investigation. These included (*a*) growth and feed consumption, (*b*) increase in weight of the thyroid gland, (*c*) uptake of radioiodine by the thyroid gland, (*d*) binding of radioiodine in organic combination, and (*e*) urinary excretion of radioiodine. The correlation of these factors with the thiouracil content of the diets of the experimental rats will now be considered.

Table II

Effect of Dietary Thiouracil on Distribution of Injected Radioiodine in Rats

Group No.	Thiouracil added to diet	Weight of thyroid glands		Per cent of administered I^* recovered in whole thyroid gland				Per cent of administered I^* recovered in urine	
				As total I^*		As organic I^*			
				Uptake	Uptake per mg. gland weight per 100 gm. body weight	Uptake	Uptake per mg. gland weight per 100 gm. body weight	As total I^*	As organic I^*
	mg. per kg.	*mg.*	*mg. per 100 gm. body weight*						
1	0	9.9	9.8	12.0	1.23	8.9	0.91	57.6	14.8
2	0	9.9	11.2	14.1	1.26	9.2	0.82	57.6	14.8
3	1	9.8	10.4	15.0	1.44	9.0	0.87	58.5	23.4
4	5	9.8	10.7	14.3	1.34	6.4	0.60	49.7	16.7
5	10	8.7	9.8	11.9	1.21	7.3	0.75	50.1	19.7
6	50	10.8	12.5	8.4	0.67	8.0	0.64	44.9	10.7
7	100	13.0	15.3	10.3	0.68	6.9	0.45	57.6	11.5
8	200	14.3	17.5	3.8	0.22	3.3	0.19	62.9	13.4
9	500	20.9	22.9	3.6	0.16	2.9	0.13	39.0	12.5
10	1000	36.3	44.8	6.3	0.14	3.0	0.07	43.8	13.0
11	†	11.3	11.7	12.0	1.03	6.2	0.53	58.7	9.5
12	†	9.1	11.1	10.4	0.94	6.4	0.68	53.6	25.4
13	†	8.0	10.1	8.4	0.75	4.6	0.46	66.2	15.9
14	†	8.9	9.5	12.7	1.34	8.4	0.88	56.4	14.4
15	†	7.3	8.0	11.9	1.49	8.3	1.04	63.4	13.7

† Received dried muscle as described in Table I.

It might be expected that the depression of thyroid function in rats brought about by the ingestion of amounts of thiouracil from 0 up to 100 mg. per 100 gm. of ration would be reflected in changes in average feed consumption or in growth as measured by weight gain over the experimental period. The data in Table I indicate that there were no correlations of this type in either case.

A second measurement was the increment of thyroid gland weight with increasing dosage of thiouracil, which was employed by Turner and associates (19) as a method for determination of thiouracil. An increase in gland weight of slightly more than 3-fold was found, a sharp rise coming with levels of thiouracil greater than 10 mg. per 100 gm. of diet (Fig. 1). The gland weights were expressed in mg. per 100 gm. of body weight (Table II) and the percentage increase in gland weight over the average values for Groups 1 and 2 was plotted against the values for dietary thiouracil in Fig. 1. This measurement was insufficiently sensitive for the detection of the low levels of thiouracil present in the tissue of the hogs used in this investigation.

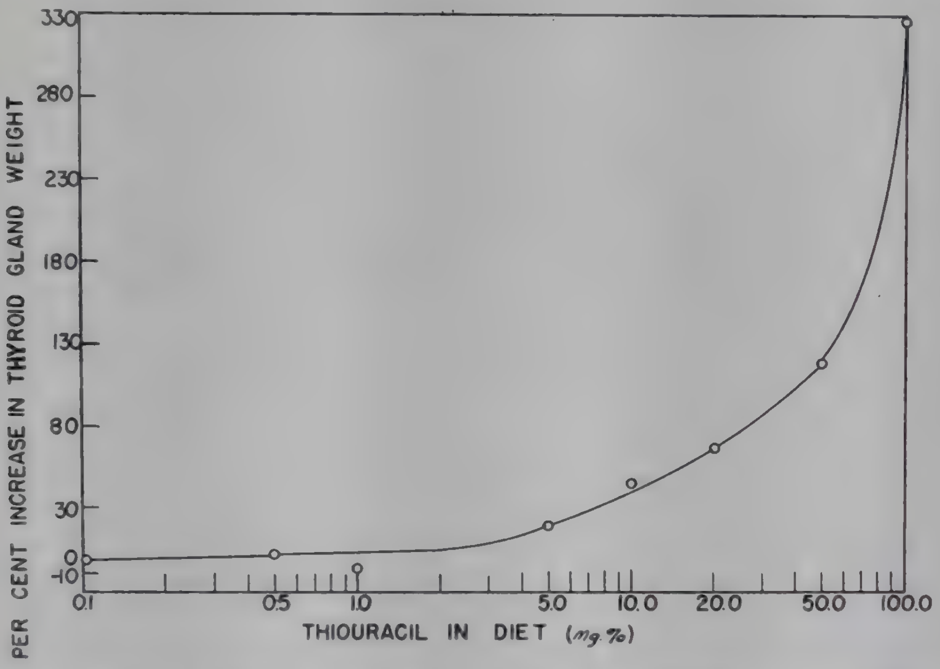

FIG. 1. Effect of dietary thiouracil on the per cent change of thyroid weight

The functional capacity of the thyroid was determined by measuring the ability of the gland to collect a tracer dose of radioiodine and to convert it to diiodotyrosine and thyroxine as shown (22) by determining the organically bound radioiodine in the glandular hydrolysate. It was found necessary to subject the data to special treatment, for, while thiouracil depresses thyroid activity, the gland is simultaneously enlarged and its ability to take up iodine is not completely blocked. Accordingly the computations showed an actual increase in the per cent of the administered dose of radioiodine accumulated in Group 10 (0.1 per cent thiouracil in the diet) as compared with Group 9 (0.05 per cent thiouracil in the diet). If, however, the calculations were made on the basis of uptake of radioiodine per mg. of gland weight per 100 gm. of body weight, plotted against the level of thiouracil in the diet, a more satisfactory response curve was obtained.

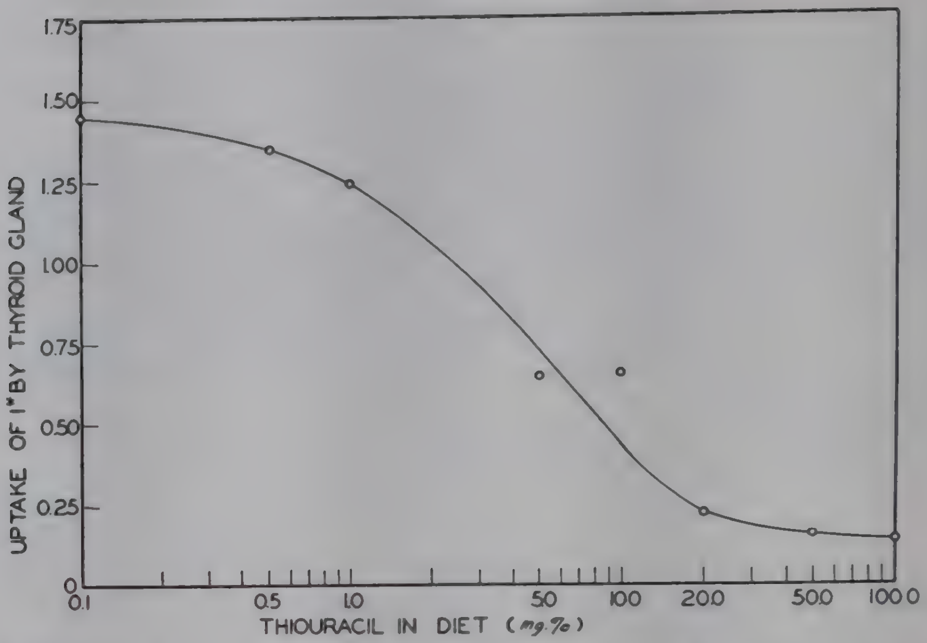

FIG. 2. Effect of dietary thiouracil on percentage uptake of radioiodine by the rat thyroid. The values for the ordinate are expressed as per cent of the administered dose of radioiodine which is taken up by the gland per mg. of gland weight per 100 gm. of body weight.

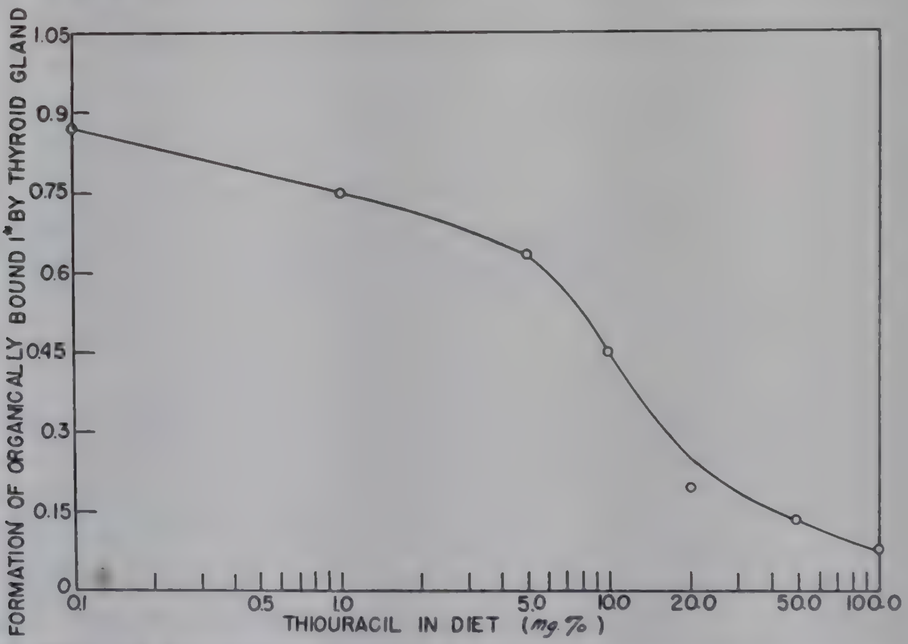

FIG. 3. Effect of dietary thiouracil on formation of organically bound radioiodine by the rat thyroid. The values for the ordinate are expressed as the per cent of the administered dose of radioiodine which is organically bound per mg. of gland weight per 100 gm. of body weight.

The curves in Figs. 2 and 3 illustrate, respectively, the course of depression of radioiodine uptake and of formation of organically bound radioiodine, with increasing dietary thiouracil.

The last measurement made was the urinary excretion of radioiodine. It was shown (23) that when radioiodine was administered to rats an average of 50 per cent of the dose was found in the urine at the end of 24 hours. Since the ingestion of thiouracil is known to interfere with the conversion of iodine to thyroxine and diiodotyrosine by the rat thyroid (21), feeding increasing amounts of thiouracil might be accompanied by the excretion of increasing amounts of radioiodine in the urine. No correlation was found, however, between dietary intake of thiouracil and urinary excretion of either total or organically bound radioiodine.

TABLE III

Thiouracil Content of Hog Tissues as Calculated from Total Radioiodine Uptake and Organic Radioiodine Formation in Rats Fed Tissues

	Tissue A				Tissue B				Tissue C	
	Goup 11		Group 12		Group 13		Group 14		Group 15	
	Total I*	Organic I*	Total I*	Organic I*	Total I*	Organic I*	Total I*	Organic I*	Total I*	Organic I*
Uptake of I* per mg. thyroid gland weight per 100 gm. body weight, %	1.03	0.53	0.94	0.68	0.75	0.46	1.34	0.88	1.49	1.04
Thiouracil concentration in rat diet from Figs. 2 and 3, *mg. %*	2.3	7.8	2.9	2.9	4.8	9.7	0.53	<0.2	<0.1	<0.1
Thiouracil concentration in fresh hog tissue,† *mg. %*	1	5	8	9	3	5	2	<0.5	<0.05	<0.05
Average thiouracil concentration in fresh hog tissue, *mg. %*	6 ± 3				2 ± 1				<0.05	

† The fresh tissues contained the following amounts of water: Tissue A, 71.6 per cent; Tissue B, 74.4 per cent; Tissue C, 74.5 per cent.

The three measurements which were finally utilized in the bioassay of thiouracil were (*a*) thyroid size, (*b*) decreased uptake of radioiodine, and (*c*) decreased formation of organic radioiodine by the thyroid. Table III shows the method of calculating the thiouracil content of fresh tissues of hogs which received thiouracil. The calculations are based on the levels of dried tissues fed in the diet and on the water content of the tissues. The final value for the thiouracil concentration in fresh hog tissue was derived from the data on two groups of animals in the case of Tissues A and B, from one group for Tissue C, and from both the total radioiodine uptake and synthesis of organically bound radioiodine in each group. The final results are given as the average values, with the mean deviation.

DISCUSSION

The assay method used in the present study is not specific for thiouracil, but by it the total amount of goitrogenic material in the tissue can be measured in terms of thiouracil. Thus, any biologically active conjugated derivatives or breakdown products of thiouracil would be included in the results obtained. The use of a specific chemical method such as one based on the use of Grote's reagent could possibly give results of incomplete biological significance because of this consideration.

The results obtained from the measurement of the amount of radioactive iodine taken up by the thyroid or the per cent of the administered dose found in the gland as organically bound radioactive iodine indicate that this measurement provides a highly sensitive method for the determination of the thiouracil level of tissues fed to rats. However, the mean deviations of the values for a single tissue measured with different levels of dried tissue and by different functions (total or organically bound radioiodine) indicate that the precision of the method is low.

The work of Chaikoff and his associates (23) indicates that the per cent of an administered dose of radioactive iodine accumulated by the thyroid is a function of the amount of total inorganic iodine present in the animal. In the present study the dietary iodine intake of the pigs in the preliminary thiouracil feeding period and the rats in the subsequent assay period was not restricted. This may account for the lower percentage uptake of radioactive iodine found in the present investigation than in the studies by Chaikoff (23).

The suggestion has been made that the thiouracil level of tissue might be evaluated by measuring the growth retardation, at least in the toxic range, of rats fed the tissue.[4] The results in Table I indicate that this is not a very satisfactory procedure.

There is considerable evidence for a rapid decrease of thiouracil in the body following its oral administration, presumed to result either from breakdown or its excretion in the urine. After the ingestion of a single dose of 1.0 gm. of thiouracil, Paschkis and coworkers (24) found that the concentration in human serum reached a maximum at 2 hours and fell to a low level in 24 hours. For the same dose they also observed up to 65 per cent excreted in the urine in the first 24 hours and an additional 10 to 15 per cent during the second 24 hours. Determinations of thiouracil in the blood of fowls fed up to 0.6 per cent thiouracil, made by Pipes and Turner (19), indicated that the highest concentrations of thiouracil in the blood

[4] Kline, E. A., personal communication.

were reached soon after feeding. After the feed was withdrawn, thiouracil concentrations were found to decline, until no trace was detected after 16 to 20 hours. Astwood (25) has recently reported on the duration of action of thiouracil in man. A dose of 500 mg. of thiouracil was effective for about 24 hours and the effect of a small dose disappeared in a few hours. Furthermore, when rats were fed 4 mg. of thiouracil daily for 7 days, the thyroid was strongly depressed, but when the thiouracil administration was discontinued the gland returned to its normal condition within 2 weeks (21).

The results of this investigation indicate that withdrawal of thiouracil from the pig ration several days before an animal is sacrificed will reduce the thiouracil level of the tissue to a value of less than 0.05 mg. per 100 gm. In view of these considerations it does not seem probable that any deleterious effects could be caused in man by the ingestion of meat from thiouracil-treated swine if the material is withdrawn from the feed of the animal several days before it is sacrificed. Similar experimental work is in progress with poultry.

SUMMARY

A biological method is described for the determination of thiouracil in tissue. The procedure is based on the depression of thyroid function by thiouracil as measured by radioactive iodine when administered to rats which have been fed with the tissues to be assayed.

A standard response curve was obtained by adding various levels of thiouracil to the diet of rats and then determining the distribution of a tracer dose of radioactive iodine in these animals. When the dried muscular tissue from pigs fed 0.2 per cent thiouracil for 34 days was fed to rats, the degree of depression of thyroid function indicated a thiouracil level of 6 ± 3 mg. per cent in the fresh tissue. The withdrawal of the thiouracil supplement from the ration of the pigs for 1 and 3 days reduced the thiouracil level in the muscular tissue to 2 ± 1 and less than 0.05 mg. per cent, respectively.

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ISOLATION AND IDENTIFICATION OF HYDROLECITHIN (DIPALMITYL LECITHIN) FROM BRAIN AND SPLEEN*

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Hydrolecithin (dipalmityl lecithin) has recently been isolated by Thannhauser, Benotti, and Boncoddo from beef lung (1). The separation of this ether-insoluble phospholipide from sphingomyelin was achieved by a sequence of fractional extractions under control of an analytical method which is based on the observation that sphingomyelin is resistant to N sodium or potassium hydroxide at 37° in contrast to hydrolecithin which is saponified under these conditions (2). Finally, an ether-insoluble substance was obtained which was completely saponifiable by N sodium hydroxide at 37° and identified as dipalmityl lecithin.

The present paper deals with the application of this procedure to the lipides of brain and spleen. It will be demonstrated that hydrolecithin is present not only in the sphingomyelin fraction of lung, but also in brain and spleen, and that the hydrolecithin isolated from brain and spleen is identical with dipalmityl lecithin. The presence of large amounts of cerebrosides in brain necessitated the modification of the preparative procedure applied for the isolation of hydrolecithin from lung.

Preparation of Hydrolecithin from Brain

Batches not exceeding 25 pounds of beef brain were used for each single preparation, since the abundance of cholesterol and cerebrosides in this organ caused great practical difficulties which are not encountered in other material such as lung and spleen. These difficulties increase when the isolation is undertaken on a larger scale.

The fresh beef brains are minced, washed twice with acetone, filtered, and dried in a vacuum dryer at 60°. The material is then ground to a powder and extracted with hot acetone for 3 days in a large continuous extractor (3) in order to remove the bulk of the fat, cholesterol, and a part of the cerebrosides and monoaminophosphatides. The acetone extract is discarded despite the fact that it contains small amounts of hydrolecithin and sphingomyelin. The powder is continuously extracted with ether for 3 days. The crude ether extract is placed in the refrigerator and filtered

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on the next day with the aid of Hyflo filter aid (Johns-Manville). The filtered lipide mixture is reextracted with ether in a Soxhlet extractor for several days in order to complete the removal of the unsaturated monoaminophosphatides, cholesterol, fat, and small amounts of ceramides.

The residue (143 gm.) in the thimble is then taken up in about 1000 cc. of petroleum ether-methanol (9:1). The emulsion formed due to the presence of the Filter-Cel and cerebrosides is broken up by the addition of 1000 cc. of methanol. After standing overnight the precipitate, which consists essentially of cerebrosides and Filter-Cel, is filtered off. The filtrate is concentrated to a small volume of a thin syrupy consistency and precipitated with a large excess of acetone (1 to 2 liters). The suspension is placed in the refrigerator overnight. The precipitate (52 gm.) consists mainly of sphingomyelin, hydrolecithin, and still appreciable amounts of cerebrosides. This lipide mixture contains 2.4 per cent P of which 37 per cent is saponifiable P. (Saponifiable P represents saturated monoaminophosphatides (4).)

The material is treated with 10 volumes of glacial acetic acid (520 cc. for 52 gm.), which extracts the bulk of the phosphatides. On slight warming the major portion of the precipitate dissolves. The suspension is allowed to stand overnight at room temperature and is then filtered. The insoluble residue is extracted again with 10 volumes of acetic acid in order to extract the retained phosphatides. The glacial acetic acid filtrates are pooled and concentrated to a very small volume (viscous consistency) and precipitated with an excess of acetone (1000 to 2000 cc.). The suspension is allowed to stand overnight in the refrigerator and is then filtered. The precipitate (Substance A, 33 gm.) contains essentially sphingomyelin, small amounts of hydrolecithin and cerebrosides, and traces of amino acids.

The filtrate is concentrated to dryness under reduced pressure and dried over sodium hydroxide in an evacuated desiccator in order to remove the acetic acid as completely as possible. The residual substance is then suspended in an excess of acetone and is left overnight in the refrigerator. The precipitate is filtered (3.5 gm.). The analysis of this substance shows that 90 per cent of its total P is saponifiable P (hydrolecithin). This substance is recrystallized from a mixture of acetone-glacial acetic acid (40:1) (1 gm. of substance in 20 cc. of solution). The amount of hydrolecithin present in the ether-insoluble phospholipide fraction is approximately 25 per cent of its sphingomyelin content. Yield of hydrolecithin, 4 gm. from 25 pounds of fresh beef brains.

Physical Properties of Hydrolecithin from Brain—The substance is a white crystalline powder soluble in alcohol and glacial acetic acid, very slightly soluble in acetone, and insoluble in ether. The substance melts between 238–240°. (The mixed melting point with hydrolecithin from lung gave no depression.) The iodine number is 5.65.

Analysis—Calculated, N 1.86, P 4.1; found, N 1.81, P 4.15

The specific rotation was +6.25° (4 per cent solution of hydrolecithin in a mixture of chloroform-methanol, 1:1).

Products of Alkaline Hydrolysis of Hydrolecithin. Barium Salts of Fatty Acids—2.5 gm. of the substance were refluxed for 4 hours with saturated aqueous $Ba(OH)_2$. After cooling, the barium soaps were filtered and shaken with H_2O to remove soluble impurities. After filtering and drying, the crude barium salt of the fatty acid weighed 2.07 gm.

Identification of Fatty Acid by Vacuum Distillation of Methyl Ester—2 gm. of barium soaps were refluxed in a 5 per cent methanolic solution of H_2SO_4 for 4 hours on a water bath. The $BaSO_4$ formed was removed by filtration, and the methyl esters were extracted from the acid methanolic solution with petroleum ether. The dry weight was 1.25 gm.

The methyl esters were distilled at a pressure of 0.0015 to 0.002 mm., the substance distilling at a fairly constant temperature (94–96°), indicating the presence of only one methyl ester. The free acid was recrystallized at −15° to a constant melting point, 61° (palmitic acid, 62°); iodine number 0; mol. wt. 257 (palmitic acid, 256).

Choline—Choline, total P, and glycerophosphate were determined in the filtrate (150 cc.) of the alkaline hydrolysis. Choline was determined by the reineckate method in 1 cc. of the filtrate. Found, 345 mg. of choline; calculated for 2.5 gm. of dipalmitolecithin, 403 mg. A 1 cc. aliquot was analyzed for total P; found, 88.65 mg. of total P; calculated for 2.5 gm. of dipalmitolecithin, 103.0 mg. Both findings represent an 86 per cent recovery.

Barium Glycerophosphate—The remainder of the filtrate (145 cc.) was used for the isolation of glycerophosphate. After neutralization with acetic acid, basic lead acetate was added in slight excess. The precipitate was filtered and carefully washed with water. The lead salt was decomposed with hydrogen sulfide. The filtrate from the lead sulfide was concentrated to a small volume. A hot saturated solution of barium hydroxide was added until pH 9 was obtained. The barium salt was precipitated by the addition of 3 volumes of alcohol.

The barium salt was analyzed for glycerol (method of Blix) and for P (method of Fiske and Subbarow).

$C_3H_6O_6PBa$. Calculated. Glycerol 30, P 10
Found. " 28.8, " 9.71

The physical properties of the substance as well as the analysis of the split-products obtained by its alkaline hydrolysis show that hydrolecithin (dipalmityl lecithin) from brain is identical with dipalmityl lecithin isolated from lung.

Isolation of Hydrolecithin from Spleen

The method employed for the isolation of hydrolecithin from the ether-insoluble phosphatide mixture is essentially the same as previously reported for its isolation from lung (1).

Physical Properties of Hydrolecithin—Hydrolecithin isolated from spleen is a white crystalline substance soluble in alcohol and glacial acetic acid, very slightly soluble in acetone, and insoluble in ether. The substance melts between 238–240°. A mixed melting point with hydrolecithin obtained from lung and brain showed no depression of the melting point; iodine number 3.81.

Analysis—Calculated, N 1.86, P 4.1; found, N 1.91, P 3.98

Specific rotation, +6.25° (4 per cent solution of hydrolecithin in a mixture of chloroform-methanol, 1:1).

The amount of hydrolecithin present in spleen is approximately 25 to 40 per cent of its sphingomyelin content. Hydrolecithins (dipalmityl lecithin) isolated from spleen, brain, and lung are identical in their physical properties.

DISCUSSION

In the paper on hydrolecithin from lung it has already been pointed out that this substance (dipalmityl lecithin) is not identical with hydrolecithin prepared by hydrogenation with colloidal palladium from unsaturated lecithins of egg yolk (4–6) and of brain (7). It is also not identical with the synthetic distearyl lecithin of Grün and Limpächer (8, 9). It seems to be identical with the hydrolecithin isolated by Lesuk and Anderson (10) from *Cysticercus* larvae.

The same statements are pertinent for hydrolecithin (dipalmityl lecithin) from brain and spleen described in this paper, since hydrolecithin from these organs is identical with hydrolecithin from lung.

The identity of the hydrolecithins isolated from different organs (brain, spleen, lung) contrasts with the non-identity of the sphingomyelins isolated from these organs, since the fatty acids of brain sphingomyelin are very different from those of lung and spleen sphingomyelin.

SUMMARY

A method of isolation of crystalline hydrolecithin from beef brain is described.

The isolated saturated lecithin is dipalmityl lecithin and is identical with lung hydrolecithin.

Hydrolecithin was also isolated from spleen according to the procedure

applied for its isolation from lung. It is identical with dipalmityl lecithin prepared from brain and lung.

The yield of hydrolecithin in brain is approximately 4 gm. from 25 pounds of fresh beef brain, corresponding to 25 to 40 per cent of the sphingomyelin yield.

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THE CHEMICAL NATURE OF THE FATTY ACIDS OF BRAIN AND SPLEEN SPHINGOMYELIN. THE OCCURRENCE OF SATURATED AND UNSATURATED SPHINGOSINES IN THE SPHINGOMYELIN MOLECULE*

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Sphingomyelin prepared with the older methods from brain and spleen yielded on acid hydrolysis palmitic, stearic, and lignoceric acids (1, 2). Merz found that in addition to these saturated acids nervonic acid is present in the sphingomyelin of brain (3).

The presence of palmitic acid in the sphingomyelin molecule of brain seemed questionable, since it was shown that sphingomyelin prepared by the usual method is a mixture of hydrolecithin (dipalmityl lecithin) and sphingomyelin.

It is the purpose of this paper to describe a procedure for the preparation of sphingomyelin free of hydrolecithin from brain and spleen, and to reinvestigate its component fatty acids. In pure *sphingomyelin of brain*, stearic acid, lignoceric acid, and its unsaturated compound, nervonic acid, are present. No palmitic acid was found. Stearic acid and nervonic acid are present in larger quantities than is lignoceric acid.

Sphingomyelin free of hydrolecithin was prepared from spleen according to the method previously described for the isolation of pure sphingomyelin from lung (4). The fatty acids present in the *sphingomyelin of spleen* are the same as those found in sphingomyelin of lung; namely, palmitic acid and lignoceric acid in about equal amounts.

Carter and coworkers (5, 6) have shown that sphingosine present in the cerebroside molecule is partly saturated (hydrosphingosine) and partly unsaturated sphingosine. The findings of Carter prompted us to isolate sphingosine after acid hydrolysis of sphingomyelin and to investigate whether or not saturated sphingosine is, as in cerebrosides, also a constituent of the sphingomyelin molecule.

Crystalline sphingosine sulfate was isolated from pure sphingomyelin of brain and spleen. In both types of sphingomyelins the isolated sphingosine sulfates had iodine numbers which were much lower than that calculated for unsaturated sphingosine sulfate. It seems therefore justified to assume

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that the sphingosine groups not only of the cerebrosides but also of the sphingomyelins are mixtures of hydrosphingosine and sphingosine.

EXPERIMENTAL

Preparation of Sphingomyelin from Brain by Alkaline Saponification

Two batches of crude sphingomyelin from brain (16 gm. each) obtained from the ether-insoluble lipide fraction by fractionation with glacial acetic acid-acetone mixture, described in the preceding paper as Substance A (7), were separately suspended in a small amount of water and ground to a paste. To each, 320 cc. of 0.25 N sodium hydroxide were added. These suspensions were shaken at 37° for 4 to 5 days. After acidification with glacial acetic acid they were both placed in the refrigerator and left there overnight.

Despite the acidification, the insoluble sphingolipides were present partially in the form of an emulsion which could not be filtered. In order to obtain a filtrable precipitate it was necessary to add an equal volume of acetone. This suspension was filtered over a thin layer of Hyflo filter aid.

The precipitates of the two batches were washed with acetone, pooled, and extracted with ether in a Soxhlet apparatus for 2 to 3 days for the purpose of removing the fatty acids. The contents of the thimble were taken up in petroleum ether-methanol (9:1) and filtered. The filtrate was concentrated to a very small volume and the phosphatide was precipitated almost quantitatively with an excess of acetone. The dried precipitate (25.5 gm.) was dialyzed against running water for 24 hours for the purpose of removing inorganic material. The filtration of the dialyzed suspension was difficult in spite of the Hyflo filter aid. It was expedited upon addition of an equal volume of acetone and acidification of the suspension with dilute HCl. The dried precipitate containing traces of cerebrosides was taken up in petroleum ether-methanol (9:1) and run through a column of Al_2O_3 for the selective adsorption of cerebrosides (8). The sphingomyelin was recovered from the concentrated solution by precipitation with acetone (16 gm.). It was recrystallized from 800 cc. of hot ethyl acetate to which 8 cc. of methanol were added. The following results were obtained upon analysis of this substance, which is free of hydrolecithin: 3.28 per cent N, 3.76 per cent P (N:P ratio 2:1).

Properties of Sphingomyelin from Brain—Brain sphingomyelin is a white crystalline substance easily soluble in benzene, soluble in warm alcohol and hot ethyl acetate. It is insoluble in ether and acetone. The substance discolors at 190–195° and melts between 212–213°. $[\alpha]_D^{22} = +6.25°$ (4 per cent solution of sphingomyelin in a mixture of chloroform-methanol, 1:1); iodine number, 42.5.

Products of Acid Hydrolysis of Sphingomyelin from Brain. Fatty Acids—

Two portions, each 6 gm., of sphingomyelin (hydrolecithin-free) were refluxed in separate flasks each containing 200 cc. of a solution of 10 per cent sulfuric acid in methanol. After cooling, the methyl esters were extracted several times with petroleum ether. The petroleum ether fractions from both flasks were pooled, washed once with water, and concentrated to dryness. Weight of the methyl esters, 5.2 gm. The methyl esters were taken up in 10 volumes of absolute alcohol (52 cc. for 5.2 gm.) and allowed to stand at room temperature for 3 hours. The resulting precipitate, Fraction I, was filtered and dried; weight 0.65 gm.

The mother liquor was left in the refrigerator overnight. The precipitate, Fraction II, was filtered and dried; weight 1.4 gm. The final mother liquor was concentrated to a very small volume of an oil. A small amount of white solid material which settled out from the oil while drying in the desiccator was separated. The oil, Fraction III, weighed 2.55 gm. The white material, Fraction IIIA, weighed 0.25 gm. The iodine numbers of the three main fractions were 8.1 (Fraction I), 9.05 (Fraction II), 45.5 (Fraction III). These figures indicate the presence of an unsaturated fatty acid, most of which appears in Fraction III.

Main Fraction I yielded two fractions, Fraction IA (solid) distilling from 91.5–106° at 0.001 mm. pressure, and Fraction IB (semisolid) distilling from 90–96° at 0.001 mm. pressure. Fraction IA was converted to the free acid after saponification with 1 cc. of N aqueous sodium hydroxide in 15 cc. of methanol. The acid was recrystallized several times from petroleum ether at room temperature; m.p. 80°; mol. wt. by titration, with 0.1 N sodium ethylate, 372; iodine number 2.56. These figures are in close agreement with the properties of lignoceric acid (m.p. 82°, mol. wt. 368). Fraction IB was converted to the free acid and found to contain some lignoceric acid, which was not further purified.

Main Fraction II yielded two fractions, Fraction IIA (solid) distilling from 70–87° at 0.001 mm. pressure, and Fraction IIB (semisolid) distilling from 69–97° at 0.0075 mm. pressure. Fraction IIA was converted to the free acid as described above and recrystallized several times from a small volume of petroleum ether at room temperature; m.p. 69°; mol. wt. by titration, 282; iodine number 2.61. These results indicate that the fatty acid is stearic acid (m.p. 69°, mol. wt. 284). Fraction IIB, a small part of the main fraction, was a mixture of lignoceric and stearic acids.

Main Fraction III was converted directly to the fatty acids by saponification with 0.1 N alcoholic sodium hydroxide[1] and acidification with dilute hydrochloric acid. The fatty acids were taken up in 40 cc. of petroleum ether and placed in an insulated container which was kept at −10° by means of dry ice. After 30 minutes the saturated acids had settled. The unsaturated acid in the filtrate was remethylated and distilled at a pressure of 0.001 mm. Two fractions were obtained, Fractions IIIA and IIIB.

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[1] In the saponification of nervonic acid alcoholic sodium hydroxide is used rather than aqueous sodium hydroxide because the nervonic acid is not completely saponified in an aqueous solution.

Fraction IIIB was converted to the free acid as described above and recrystallized from 5 cc. of petroleum ether at −10°; m.p. 41.5°; mol. wt., by titration with 0.1 N sodium ethylate, 367; iodine number 66. Nervonic acid, m.p. 41.5; mol. wt. 366; iodine number 69.

Fraction IIIA contained small amounts of stearic acid and nervonic acid.

The nervonic acid from Fraction IIIB was hydrogenated for 4 hours with palladium charcoal as a catalyst. The hydrogenated substance melted at 81.5°. Lignoceric acid, m.p. 82°. This melting point substantiates the fact that the unsaturated acid is nervonic acid which was converted by hydrogenation to lignoceric acid.

In another experiment Adams' PtO_2 (obtained from the American Platinum Works, Newark, New Jersey) was used as a catalyst in the hydrogenation of nervonic acid but did not prove to be so satisfactory as the palladium charcoal.

The fractionation of the fatty acids from brain yielded lignoceric, stearic, and nervonic acids in the approximate proportions of 1:2:2.

Isolation of Sphingosine Sulfate (*Sphingosine-Hydrosphingosine Mixture*)—After the removal of the fatty acid esters, the acid hydrolysate of the spingomyelin was neutralized with 4 N alcoholic KOH. The potassium sulfate was filtered off and the filtrate was made just acid to litmus with glacial acetic acid. The solution was made strongly alkaline with potassium hydroxide and the sphingosine was extracted with ether. The ether extract was washed with water and dried over Na_2SO_4.

The iodine number of this crude base was 41.5. It was recrystallized from petroleum ether at room temperature. The insoluble Fraction A was filtered and the mother liquor was left in the refrigerator overnight. The precipitate (Fraction B) was filtered. The iodine numbers of Fractions A and B were 46 and 19.4 respectively.

0.85 gm. of Fraction A was converted to the sulfate (weight 0.94 gm.). It was recrystallized from 25 cc. of hot absolute alcohol and allowed to stand at room temperature. The precipitate (0.6 gm.) had an iodine number of 46. (The theoretical iodine numbers are 72.9 for sphingosine sulfate, 0 for dihydrosphingosine sulfate.)

$(C_{18}H_{37}NO_2)_2 \cdot H_2SO_4$ and $(C_{18}H_{39}NO_2)_2 \cdot H_2SO_4$. Calculated, N 4.02
Found, " 3.82

After repeated fractionations of the sphingosine sulfate-dihydrosphingosine sulfate mixture from absolute alcohol, we finally obtained a sphingosine sulfate preparation which had an iodine number of 60.

Preparation of Dihydrosphingosine Sulfate—50 mg. of recrystallized Fraction A (iodine number 60) were dissolved in 50 cc. of glacial acetic acid and hydrogenated for 4 hours with PtO_2 as a catalyst. The iodine number of the hydrogenated product was 4.9. Fraction B of the crystallized sphingosine sulfate (iodine number 19.4) was also hydrogenated under the same conditions and yielded a substance with an iodine number of 4.4.

We were unable to make the derivatives of sphingosine and dihydrosphingosine described by Carter and his associates (6) because of the lack of sufficient material. However, the isolation of the two fractions of sphingosine sulfate with respective iodine numbers of 60 and 19.4 and the conversion of both to hydrosphingosine sulfate by hydrogenation justify the assumption that sphingosine sulfate obtained after hydrolysis of sphingomyelin is a mixture of sphingosine sulfate and dihydrosphingosine sulfate.

Preparation of Pure Sphingomyelin from Beef Spleen

The method employed for the isolation of hydrolecithin and the preparation of pure sphingomyelin from the ether-insoluble phosphatide mixture from spleen is essentially the same as that previously reported for lung (4, 9). The yield of sphingomyelin from 50 pounds of spleen was 6.5 gm.

The analysis of the recrystallized substance gave the following results: 3.18 per cent N, 3.77 per cent P (N:P ratio 2:1).

Properties of Sphingomyelin from Spleen—Spleen sphingomyelin is a white crystalline substance soluble in benzene, warm alcohol, and hot ethyl acetate, insoluble in ether and acetone. It can be easily recrystallized from a large volume of hot ethyl acetate to which a few cc. of methanol have been added; m.p. = 217°; $[\alpha]_D^{22} = +6.25°$ (4 per cent solution of sphingomyelin in a mixture of chloroform-methanol, 1:1); iodine number 33.5.

Fatty Acids of Pure Sphingomyelin Prepared from Spleen—4.3 gm. of sphingomyelin obtained by the procedure as described for lung (4) were refluxed for 4 hours with 143 cc. of 10 per cent sulfuric acid in methanol. After cooling, the methyl esters were extracted with three portions of petroleum ether. The pooled extracts were concentrated to dryness. The methyl esters weighed 1.7 gm.

On redistillation of the methyl esters a liquid fraction and a solid fraction were obtained. The liquid fraction distilled from 85–96.5° at a pressure of 0.001 mm., while the solid fraction distilled from 90–109.6° at a pressure of 0.0025 mm.

The liquid fraction was converted to the free acid by saponification with 1 cc. of aqueous sodium hydroxide in 15 cc. of methanol. The acid was recrystallized from petroleum ether several times at 0°; m.p. 61°; mol. wt., by titration with 0.1 N sodium ethylate, 264; iodine number 0. Palmitic acid, m.p. 62°; mol. wt. 256. It is evident that the fatty acid is palmitic acid.

The solid fraction was converted to the free acid as described above. The acid was recrystallized from petroleum ether several times at room temperature; m.p. 80°; mol. wt. by titration 385; iodine number 0. Lignoceric acid, m.p. 82°; mol. wt. 368.

Sphingosine sulfate was isolated in crystalline form from acid hydrolysis of sphingomyelin as described for brain sphingomyelin. The iodine number of this preparation was 36.7 (iodine number of unsaturated sphingosine 72.9). After hydrogenation with PtO_2 (Adams' catalyst) this substance gave an iodine number of 0.

DISCUSSION

Sphingomyelin of brain is different in regard to its component fatty acids from the sphingomyelin of visceral organs like lung and spleen.[2] In brain sphingomyelin an unsaturated fatty acid, namely nervonic acid, is found in larger amounts, while its saturated compound, lignoceric acid, is present in smaller quantities than in other organs.

Palmitic acid is not present in sphingomyelin of brain after it is freed of hydrolecithin (dipalmityl lecithin), while in the visceral organs palmitic acid is found together with lignoceric acid as the only fatty acid constituents of sphingomyelin.

Stearic acid is present together with nervonic acid as the main constituent fatty acid of brain sphingomyelin. It has not been found in the sphingomyelin of other organs.

Sphingomyelin of spleen contains palmitic acid and lignoceric acid in equal quantities. Its fatty acid components are thus essentially the same as those of lung sphingomyelin.

The physiological significance of the difference of the component fatty acids of brain sphingomyelin and the sphingomyelin of visceral organs is not known.

The sphingosine fraction of the hydrolysate was isolated from brain as well as from spleen sphingomyelin. The iodine number of the apparently uniformly crystallized sphingosine sulfate of both organs showed that this substance is a mixture of hydrosphingosine and unsaturated sphingosine. The sphingosine groups of the sphingomyelin thus consist of saturated and unsaturated sphingosine, in analogy to the composition of the cerebrosides as previously shown by Carter and his associates (5, 6).

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[2] Brain is an ectodermal organ. Visceral organs like lung and spleen are of endodermal and mesenchymal origin.

SUMMARY

1. A procedure for the preparation of pure brain sphingomyelin free of hydrolecithin is described.

2. The fatty acids present in brain sphingomyelin are different from those present in other organs investigated. The component fatty acids of brain sphingomyelin are stearic, nervonic, and lignoceric acids, while lignoceric acid and palmitic acid are the component fatty acids of sphingomyelin prepared from spleen and lung.

3. Sphingosine isolated after acid hydrolysis of brain as well as spleen sphingomyelin is a mixture of hydrosphingosine and unsaturated sphingosine.

The authors are indebted to Dr. Gerhard Schmidt for his helpful suggestions.

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MICROBIOLOGICAL DETERMINATION OF HISTIDINE IN PROTEINS AND FOODS

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Leuconostoc mesenteroides P-60, *Lactobacillus fermenti* 36, and *Streptococcus faecalis* have all been used in the assay of histidine (1–11). For the work reported in this paper both *L. mesenteroides* P-60[1] and *S. faecalis* 9790[1] were used. The standard curve obtained with *S. faecalis* 9790, although having a dip in the middle, was adequate in every respect for accurate assays of histidine. As a check, a number of foods were assayed with *L. mesenteroides*.

EXPERIMENTAL

Organisms and Basal Media—*Leuconostoc mesenteroides* P-60 was used with the medium reported for lysine (12), with the exception that pyridoxine was replaced with pyridoxamine (400 γ per liter). *Streptococcus faecalis* 9790 was used with the medium reported for threonine (13).

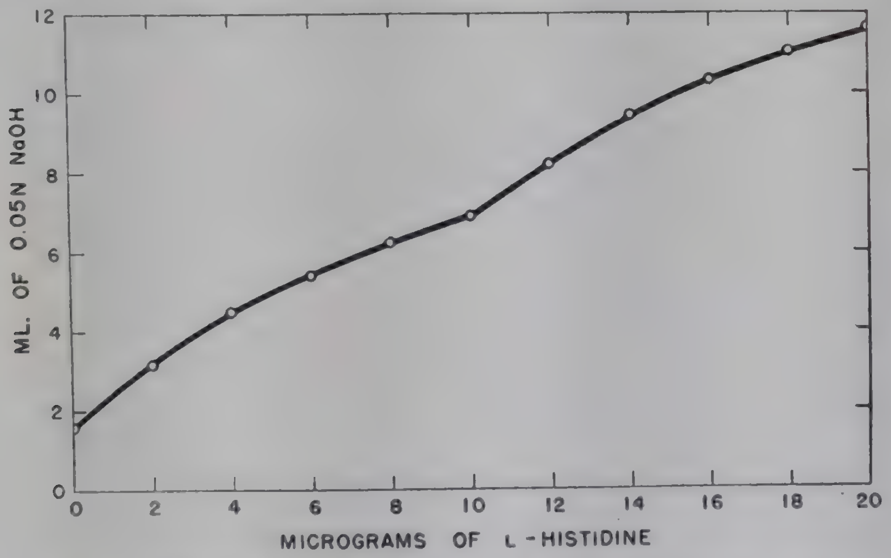

FIG. 1. Standard curve for histidine with *Streptococcus faecalis*

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[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

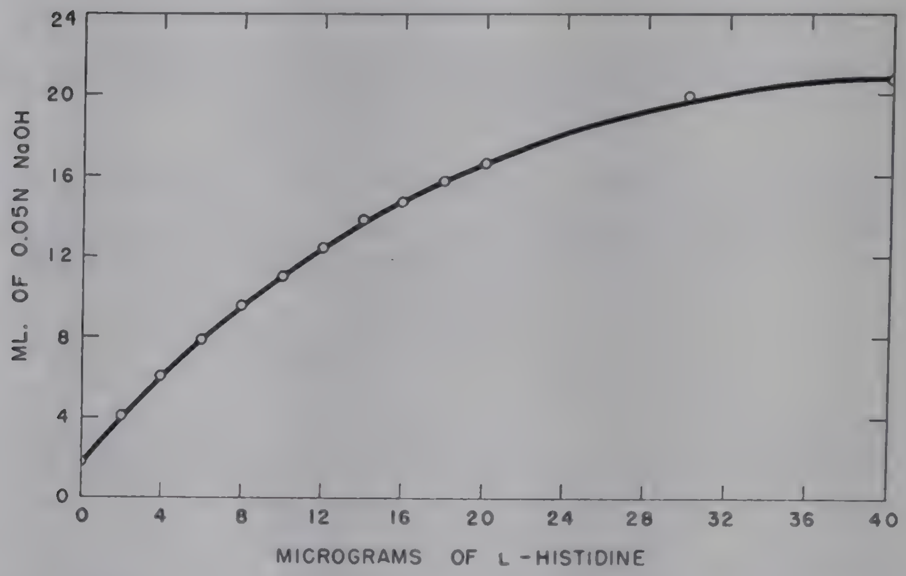

FIG. 2. Standard curve for histidine with *Leuconostoc mesenteroides*

TABLE I

Recovery of Histidine Added to Protein Hydrolysates

Protein hydrolysate	Histidine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	*per cent*
Corn, whole, yellow	1.35	4	5.35	5.35	100
	2.70	4	6.70	6.70	100
	4.05	4	8.05	8.10	101
Edestin	2.50	2	4.50	4.50	100
	2.50	4	6.50	6.30	97
	2.50	6	8.50	8.60	101
	2.50	8	10.50	10.50	100
Glycinin	1.70	2	3.70	3.70	100
	3.40	2	5.40	5.30	98
	5.10	2	7.10	7.10	100
	6.80	2	8.80	9.20	105
Soy bean flour	2.30	2	4.30	4.30	100
	2.30	4	6.30	6.10	96
	2.30	6	8.30	8.30	100
	2.30	8	10.30	10.30	100
Wheat, whole	1.60	2	3.60	3.60	100
	1.60	4	5.60	5.50	98
	1.60	6	7.60	7.40	97
	1.60	8	9.60	9.60	100

* Not corrected for moisture and ash.

Assay Procedure—The procedures followed for the cultures, inoculum and preparation of samples were identical with those described in a previous paper (14), with the exception that 0.05 N alkali was used for the titrations.

Preparation of Histidine Standards—L-Histidine solutions ranging in concentration from 2 to 20 γ per ml. were used in preparing the standard curves. The titration values of these curves (Figs. 1 and 2) were not altered by the addition of 1.2 mg. of any of the nineteen amino acids to the

TABLE II

*Histidine Content of Some Proteins and Foods Determined at Different Assay Levels**

Protein assay level	Histidine found									
	Lactalbumin		Cottonseed flour		Whole dried egg		Black-eyed peas		White rice	
γ	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cent*	γ	*per cent*
100	1.40	1.40								
200	2.80	1.40	2.90	1.45	2.10	1.05				
300	4.20	1.40								
400	5.70	1.42	6.00	1.50	4.20	1.05				
500							3.30	0.66		
600			8.50	1.42	6.20	1.03				
800			11.20	1.40	8.40	1.05				
1000							6.70	0.67	1.65	0.17
1500							10.00	0.67		
2000							13.30	0.67	3.30	0.16
3000									4.90	0.16
4000									6.50	0.16
Average..		1.40		1.44		1.05		0.67		0.16

* Not corrected for moisture and ash.

TABLE III

*Comparison of Histidine Content by Leuconostoc mesenteroides and Streptococcus faecalis**

Material	N	Histidine content	
		L. mesenteroides	*S. faecalis*
	per cent	*per cent*	*per cent*
Casein	16.07	2.50	2.46
Corn, whole, yellow	2.22	0.27	0.26
Cottonseed flour	10.36	1.42	1.44
Edestin	18.55	2.46	2.32
Glycinin	17.30	1.80	1.83
Ovalbumin	15.98	1.93	1.93
Peanut flour	10.15	1.19	1.20
Rice, white	1.26	0.16	0.16
Soy bean flour	8.85	1.15	1.16
Wheat, whole	3.07	0.34	0.34

* Not corrected for moisture and ash.

medium. No change was obtained in the titration values of the *Streptococcus faecalis* curve (Fig. 1) when the amounts of vitamins, uracil, adenine, and guanine were increased in the medium, nor did the addition of asparagine, glutamine, ascorbic acid, pimelic acid, β-alanine, inositol, or xanthine change these values in any way.

TABLE IV

Histidine Content of Some Proteins and Foods

Percentages calculated for ash- and moisture-free material.

Material	N	Histidine	Values from literature
	per cent	*per cent*	
Arachin	18.30	2.16	2.31 (15), 2.16 (16)
Casein	16.07	2.65	2.8 (1), 3.1 (2), 3.0 (3), 2.6 (4), 3.2 (5), 4.1 (6), 2.6 (7), 2.53 (15)
Coconut globulin	17.42	1.52	2.42 (17)
Conarachin	18.20	2.05	
Cottonseed globulin	18.00	3.38	2.9 (18)
Edestin	18.55	2.50	2.62 (11), 2.52 (15), 2.8 (16)
Gelatin (Bacto)	18.32	0.77	0.58 (1), 0.79 (4), 0.83 (5), 1.0 (6), 0.9 (16)
Glycinin	17.30	1.97	2.08 (15)
Lactalbumin	15.39	1.50	1.8 (4), 1.7 (16)
Ovalbumin (crystalline)	15.98	2.06	2.3 (1), 2.4 (5), 2.1 (16)
Ox muscle	16.00	1.98	3.5 (4), 1.9 (16), 2.25 (19)
Peanut, total globulins	18.01	1.65	
Phaseolin (navy bean)	16.07	2.24	
Wheat bran globulin	17.76	1.86	
Zein	16.00	0.76	0.85 (15), 0.7 (16)
Barley, pearled	1.86	0.21	0.19 (6)
Brazil nut meal	9.03	1.08	
Corn germ, defatted	3.93	0.67	0.71 (16)
" whole, yellow	2.22	0.31	0.44 (6), 0.33 (16), 0.29 (20)
Cottonseed flour	10.36	1.62	1.78 (6), 1.68 (16)
Egg, whole, dried	8.11	1.17	
Milk, dry, skim	6.57	1.01	0.99 (1), 1.33 (6), 0.99 (7), 0.92 (21)
Oatmeal	2.73	0.38	0.41 (6), 0.34 (16)
Peanut flour	10.15	1.34	1.33 (16)
Peas, black-eyed	4.15	0.75	
Rice, white	1.26	0.18	0.12 (16)
Rye, whole	1.98	0.27	0.22 (1)
Soy bean flour	8.85	1.32	1.27 (1), 1.65 (6), 1.55 (10)
Wheat germ, defatted	6.50	0.84	1.0 (16)
" whole	3.07	0.39	0.38 (1), 0.46 (6)
Yeast, dried, brewers'	7.71	0.78	1.0 (1), 1.38 (22)

Recovery of histidine added in different proportions to hydrolysates of whole corn, edestin, glycinin, soy bean flour, and whole wheat was satisfactory (Table I).

Table II shows histidine values for lactalbumin and several foods analyzed at different assay levels.

Table III gives histidine values of casein and three other proteins together with several foods as found by the use of two different organisms and media.

The final corrected values for histidine (Table IV) in the proteins and foods[2] assayed agree well with those reported for the same materials by others using microbiological methods. The results obtained by chemical methods, especially those of Vickery and Winternitz (15) and Block and Bolling (16), are likewise in close agreement with the values herein reported.

SUMMARY

Two microbiological methods are described for the determination of histidine. The procedures have been applied to the assay of proteins and foods with results that agree closely with those obtained by others using either microbiological or chemical methods.

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[2] The sources and preparation of the samples assayed are given in a previous publication on the determination of methionine (23).

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EXCRETION OF BENZOQUINONEACETIC ACID IN HYPOVITAMINOSIS C

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Sealock *et al.* (1) and Levine *et al.* (2) showed that keto and hydroxy derivatives of phenylalanine and tyrosine were excreted in the urine when insufficient ascorbic acid was available for the metabolic needs of the organism. Administration of the vitamin promptly prevented the appearance of these partial metabolites. Fishberg (3) showed that the urine in those pathologic states brought about by ascorbic acid deficiency became capable of inducing methemoglobin formation *in vitro*. This *in vitro* formation of methemoglobin by the urine is pathognomonic of a very rare metabolic disorder first described by Stokvis (4), "autotoxic enterogenous cyanosis," in which some substance of unknown chemical constitution circulating in the blood stream caused the formation of methemoglobin and resulted in the typical intense cyanosis.

In this investigation we have attempted to identify this substance, which was excreted in large quantities in the urine of a deeply cyanotic 55 year-old subject. The intensity of the cyanosis varied directly with the excretion in the urine of a substance, which on the basis of its chemical properties, its ultraviolet absorption spectrum, and oxidation-reduction potential appears to be benzoquinoneacetic acid, the oxidized phase of the oxidation-reduction system homogentisic-benzoquinoneacetic acid. The method of Valeur (5) for the quantitative estimation of water-soluble *p*-quinones could be applied to its quantitative determination. Also, we found that the power of methemoglobin formation *in vitro* was by no means confined to urine excreted by subjects suffering from enterogenous cyanosis. Table I shows that a methemoglobin-forming substance having identical chemical properties and physical constants could be detected in the urine excreted in those pathologic states in which diminished excretion of vitamin C in the urine had been previously reported, notably scurvy and rheumatic fever. Guinea pigs maintained on a scorbutic diet and showing manifest lesions of scurvy also excreted an identical substance. It can be seen in Table I that urine excreted in the above pathologic states showed a number of chemical reactions which could be reproduced by substituting a solution containing 50 mg. per cent of benzoquinone for the urine.

The reactions used were the following. (1) 1 cc. of 10 per cent KI and 1 cc. of 2 N H_2SO_4 were added to 2 cc. of solution. A positive reaction is indicated by a yellowish brown color, which, on addition of a few drops of soluble starch, turns deep blue. (2) 2 cc. of solution were added to 2 cc. of blood which had previously been diluted 1:4 with distilled water. A positive reaction is shown by the chocolate-brown color and the appearance of an intense absorption band at 630 mμ. (3) To 2 cc. of dimethyl-*p*-phenylenediamine in acetic acid solution, 2 cc. of the solution are added. A positive reaction is shown by the appearance of a brilliant red color. On spectrophotometric examination the curve of this red solution shows two absorption peaks at 520 and 550 mμ similar to those of the meriquinone, Wurster's red. (4) To 2 cc. of benzidine in acetic acid solution 2 cc. of the solution are added. A positive reaction is shown by a bright orange color. (5) To 1 cc. of the solution 2.8 mg. of 2,4-dinitrophenylhydrazine in 1 cc. of N HCl are added. A positive reaction is indicated by clouding of the solution within a few minutes and a yellow precipitate on standing. (6) The solution is made alkaline and allowed to remain in an incubator. After

TABLE I

Chemical Tests for p-Quinone

Solution	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7
I. 50 mg. % aqueous benzoquinone	+	+	+	+	+	+	−*
II. 50 mg. % benzoquinone in normal urine	+	+	+	+	+	+	+
III. Urine (enterogenous cyanosis)	+	+	+	+	+	+	+
IV. Distillate from (III)	+	+	+	+	+	+	+
V. Normal urine	−	+†	−	−	+‡	−	+*
VI. Distillate from (V)	−	−	−	−	−	−	−
VII. 50 mg. % phenylpyruvic acid in urine§	−	−	−	−	+	−	−
VIII. Distillate from (VII)	−	−	−	−	−	−	−
IX. Urine from scorbutic subject‖	+	+	+	+	+	+	+
X. Distillate from (IX)	+	+	+	+	+	+	+

* See the text.

† Only after 24 hours standing at 40°.

‡ Cloudiness with very slight precipitate after an hour.

§ Phenylpyruvic acid was taken as representative of the known intermediates of tyrosine metabolism, and a control solution of 50 mg. per cent was used. It was synthesized by the method of Herbst and Shemin (6).

‖ The subject had subsisted on a diet of chicken soup, chicken, and tea for 40 years. Multiple hemorrhages and all clinical signs of scurvy.

24 hours the solution turns dark brown and deposits a black precipitate. This phenomenon was noted by Mackenzie Wallis (7) in the urine of the five cases of enterogenous cyanosis reported by him in 1913. (7) A few drops of concentrated HCl are added to 5 cc. of the solution. A positive reaction is shown by the instant appearance of a warm red color. This reaction was stated by both Stokvis (4) and van den Bergh (8) to be characteristic of enterogenous cyanosis, but it may be reproduced readily by adding a few drops of concentrated HCl to normal urine in which benzoquinone has been dissolved. This reaction is negative in an aqueous solution of benzoquinone. A normal urine will show a rose color if treated with concentrated HCl and allowed to stand for 24 hours.

Crystalline benzoquinoneacetic acid was prepared. 50 cc. of cleared urine positive to the iodine-starch reaction were placed in a separatory funnel and 50 cc. of peroxide-free ether added. The quantity of 2 N HCl required to change the pH of the urine to 3.4 was determined potentiometrically. The calculated amount was added quickly and the mixture shaken vigorously. The ether became positive to the iodine-starch reaction. On evaporation of the ether, bright yellow crystals appeared, identical with those obtained by Mörner (9) by oxidation of homogentisic acid. We prepared the dimethyl ether of homogentisic acid (Wolkow and Baumann (10)) from a positive urine distillate by treatment in alkaline solution at room temperature with methyl sulfate.

870 cc. of urine containing 469 mg. of methemoglobin-forming substance (pH 5.62) were distilled. An indicator solution consisting of KI, H_2SO_4, and starch was placed in the receiver. When the indicator turned blue, the receiver was changed. A buffer solution (pH 7.17) was placed in the receiver and the distillation continued. A steam distillate (650 cc. containing 115 mg. of benzoquinoneacetic acid) was obtained. The material in the distilling flask had lost its power of liberating I from KI. The yield was less than 25 per cent. This steam distillate was concentrated at room temperature, 15 mm. pressure, pH 7.12. 100 cc. containing 98 mg. of benzoquinoneacetic acid were obtained.

This was quickly reduced with stannous chloride in alkaline solution till the iodine-starch reaction of a sample became negative. The solution was then shaken with methyl sulfate in a separatory funnel. As the methylation proceeded, the solution became warmer and the reaction turned acid to litmus. The solution was then extracted with ether. In order to hydrolyze the methyl ester of dimethylhomogentisic acid which was formed, the ethereal extract was made alkaline with 10 per cent NaOH and heated on the water bath until all of the ether was exhausted. It was then acidified with H_2SO_4 and exhausted with ether, and the ether allowed to evaporate spontaneously. The residue was dissolved in hot water, and on cooling needle-shaped crystals appeared, m.p. 123.8°.

$C_{10}H_{12}O_4$.	Calculated.	C 61.22, H 6.13
	Found.	" 61.07, " 6.18

Quantitative Determination of Methemoglobin-Forming Substance (Adaptation of Method of Valeur (5) for Determination of Water-Soluble p-Quinones)—10 cc. of 10 per cent KI and 10 cc. of 2 N H_2SO_4 are added to 20 cc. of urine. The solution turns yellowish brown if quinone is present. This is titrated with 0.01 N sodium thiosulfate, and just before the end-point, a few drops of soluble starch are added as an indicator. The end-point, the disappearance of the blue color, is extremely sharp. The number of cc. of thiosulfate × 4.2 gives the number of mg. of benzoquinoneacetic acid per 100 cc. Table II shows the day to day variation in the concentration of benzoquinoneacetic acid in the urine of our cyanotic subject.

A distillate of urine containing 55 mg. per cent of benzoquinoneacetic acid was used for the determination by the Beckman quartz spectrophotometer (H source) of the ultraviolet absorption spectrum (cell length 0.994 cm.). As can be seen from Fig. 1 the absorption spectrum was characterized by a maximum at 286 mμ and a shallow minimum between 265 and 260 mμ. When compared with the absorption curve of a distillate of the urine from a rheumatic subject, which contained 38 mg. per cent by titration, it was found that the two curves were identical. Graubner (11) found a similar ultraviolet absorption spectrum in serum, with a maximum at 285 mμ and a minimum at 270 mμ. In avitaminosis, according to Sührmann and Kollath (12), marked deviations from the normal density are encountered. Graubner found that the substance which causes this specific absorption band dissolved easily in water and alcohol but not in ether. Though unable to identify the nature of the substance, Graubner postulated a substance which contained a —C—O group or a benzene derivative. It would seem possible that benzoquinoneacetic acid formed as a normal intermediate in the catabolism of tyrosine and phenylalanine appears in the urine in sufficient quantity to give positive quinone reactions only as a result of damage to liver function, such as could be caused by lack of vitamin C.

The urine containing benzoquinoneacetic acid must show a well defined oxidation-reduction potential, that of homogentisic acid. To determine this we used a urine distillate containing 83 mg. per cent. This was titrated electrometrically with leucoindigocarmine in strongly buffered solution in an atmosphere of nitrogen with bright platinum electrodes. The nitrogen was passed over heated copper gauze before entering the electrode vessel. The technique was essentially that of Clark and Cohen (13).

TABLE II

Excretion of Benzoquinoneacetic Acid and Ascorbic Acid per 24 Hours

Day	Volume	Benzoquinoneacetic acid		Ascorbic Acid	Oxygen capacity
	cc.	*mg. per cent*	*mg.*	*mg.*	*vol. per cent*
1	410	32.9	512	1.6	15.9
2	560	48.1	250	4.8	15.8
3	870	54.0	469	13.6	16.1
4	680	47.5	323	6.0	16.2
5	1280	0.0	0	40.3	16.4
6	1180	0.0	0	47.2	17.2
7	1460	0.0	0	49.1	17.7
8	910	0.0	0	25.1	18.5
9	1240	0.0	0	32.8	17.1
10	1850	5.1	94	6.0	16.6
11	1100	9.7	106	2.7	16.7
12	1200	3.2	38	9.1	16.5
13	1140	14.1	160	3.6	16.3
14	815	20.6	168	5.2	16.5
15	845	11.9	101	7.1	16.9
16	1220	2.6	310	10.0	16.3
17	1620	16.2	262	14.9	16.7
18	1280	0.0	0	33.1	18.0
19	1230	0.0	0	23.2	18.6
20	659	22.4	149	7.2	18.0
21	1200	18.9	226	12.9	17.7
22	1520	0.0	0	31.1	18.1
23	1170	2.2	31	5.7	18.4
24	1100	0.0	0	29.5	18.3
25	980	14.2	140	7.8	17.3
26	1800	16.2	291	17.5	16.8
27	842	11.2	93	8.3	16.5
28	762	7.3	55	11.1	16.3
29	2160	5.4	116	4.4	16.7
30	2082	0.0	0	55.6	17.2
31	2052	1.9	39	113.1	18.2
32	1750	3.8	67	68.9	18.3
33	1940	0.0	0	266.1	18.4
34	1360	0.0	0	522.2	18.5
35	1000	0.0	0	480.0	18.8
36	1440	0.0	0	460.0	19.1
37	1320	0.0	0	456.3	19.5
38	2080	0.0	0	561.4	19.7

The titration was greatly facilitated by a parallel titration of the iodine liberated from acidified KI. Fig. 2 shows a typical curve at pH 7.01. The mid-point of the titration was 0.2503 volt, which is in excellent accord with the results reported by Blix (14) for homogentisic acid.

Conant and Fieser (15) have shown that hemoglobin-methemoglobin is a reversible oxidation-reduction system whose potential at body pH is approximately +0.15 volt. The potential of benzoquinoneacetic acid in its fully oxidized form can reach +0.30 volt at this pH. The formation of a quinone, however transitory its existence, has been assumed as one of the obligate intermediates in the catabolism of phenylalanine and tyrosine.

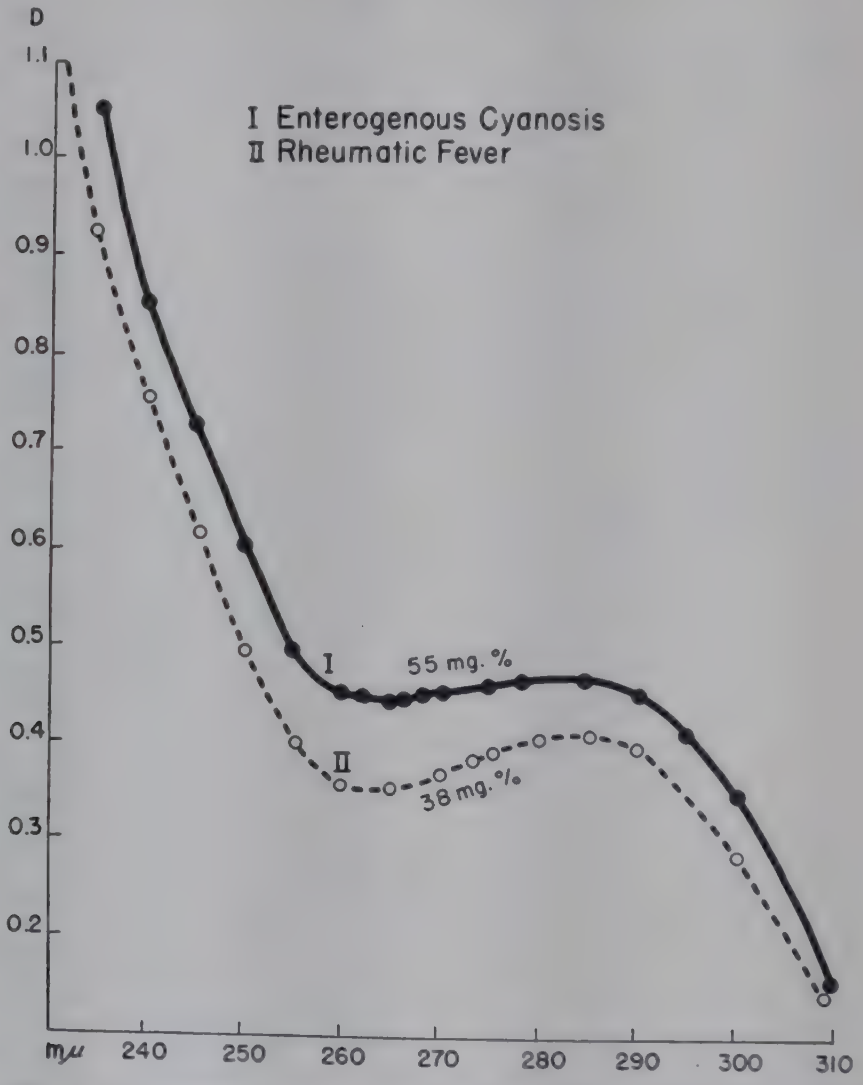

Fig. 1. Ultraviolet absorption spectrum of methemoglobin-forming substance.

Neubauer (16) postulated the conversion of tyrosine into homogentisic acid through an intermediate quinoid stage with the side chain migrating from the 4 to the 3 position in the ring. Dakin (17) found that the administration to alcaptonurics of *p*-tolylalanine and *p*-methoxyphenylalanine, *i.e.* substances incapable of forming quinoid derivatives, did not result in increased excretion of homogentisic acid. He regarded this as strong evidence that a quinoid substance is an obligate precursor of homogentisic acid. The hemoglobin of the blood stream seems capable of dealing with the transitory quinones formed during normal tyrosine catabolism, but if they appear in larger quantities or persist for a longer time, as evidenced by the appearance of benzoquinoneacetic acid in the urine, there must be a shift in the equilibrium and on this basis methemoglobin appears in the blood. It was apparent that approximately 20 per cent of the hemoglobin in the blood of our cyanotic patient had been transformed into methemoglobin. The blood volume determined by the Congo red method was 4875 cc., with a hemoglobin content of 658 gm. If the molecular weights of hemoglobin and benzoquinoneacetic acid, 68,000 and 166.6, respectively, are taken into account, it is apparent that 1.6 gm. of benzoquinoneacetic acid would have been sufficient to transform the entire hemoglobin into methemoglobin. Since 512 mg. of benzoquinoneacetic acid were excreted in the urine, some protective mechanism must have been functioning. This was the ability of hemoglobin to act as a poising agent by virtue of its middle position in the physiological oxidation-reduction scale, and thus to protect the blood against deleterious changes of E_h, with consequent danger to the oxygen transport system. The poising power is the reciprocal of the change in potential caused by the equivalent addition of an oxidant, and when 20 per cent of the hemoglobin has been transformed into methemoglobin the poising effect is 25 times as high as at 1 per cent.

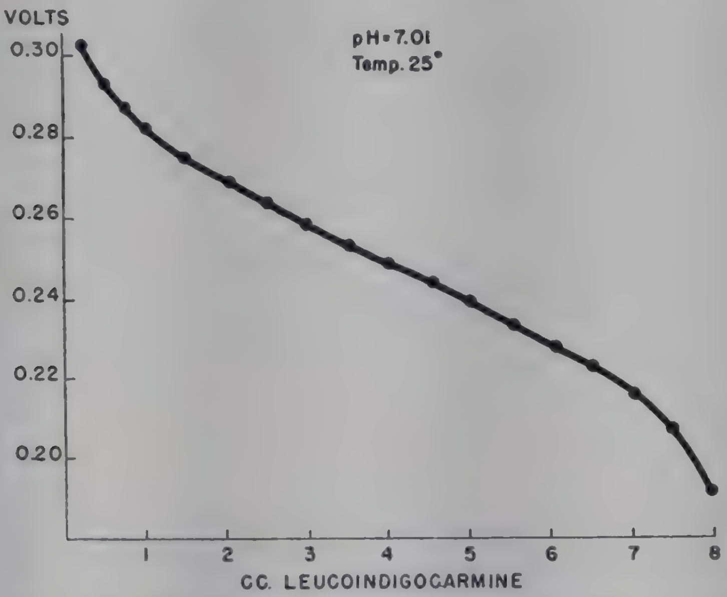

FIG. 2. Titration of methemoglobin-forming substance

Lan and Sealock (18) found that surviving slices of liver tissue from scorbutic guinea pigs had lost the faculty of complete tyrosine oxidation, but addition of vitamin C to scorbutic tissue *in vitro* resulted in the subsequent return of normal function. Fig. 3 shows that there was an inverse relationship between the amount of benzoquinoneacetic acid and the quantity of ascorbic acid excreted each day; as the former rose, the latter fell. The average quantity of 2,6-dichlorophenol indophenol-reducing substance excreted in the urine was 7.8 mg. for 20 days on a normal diet when the urine contained benzoquinoneacetic acid and 34.6 mg. for the 9 days when

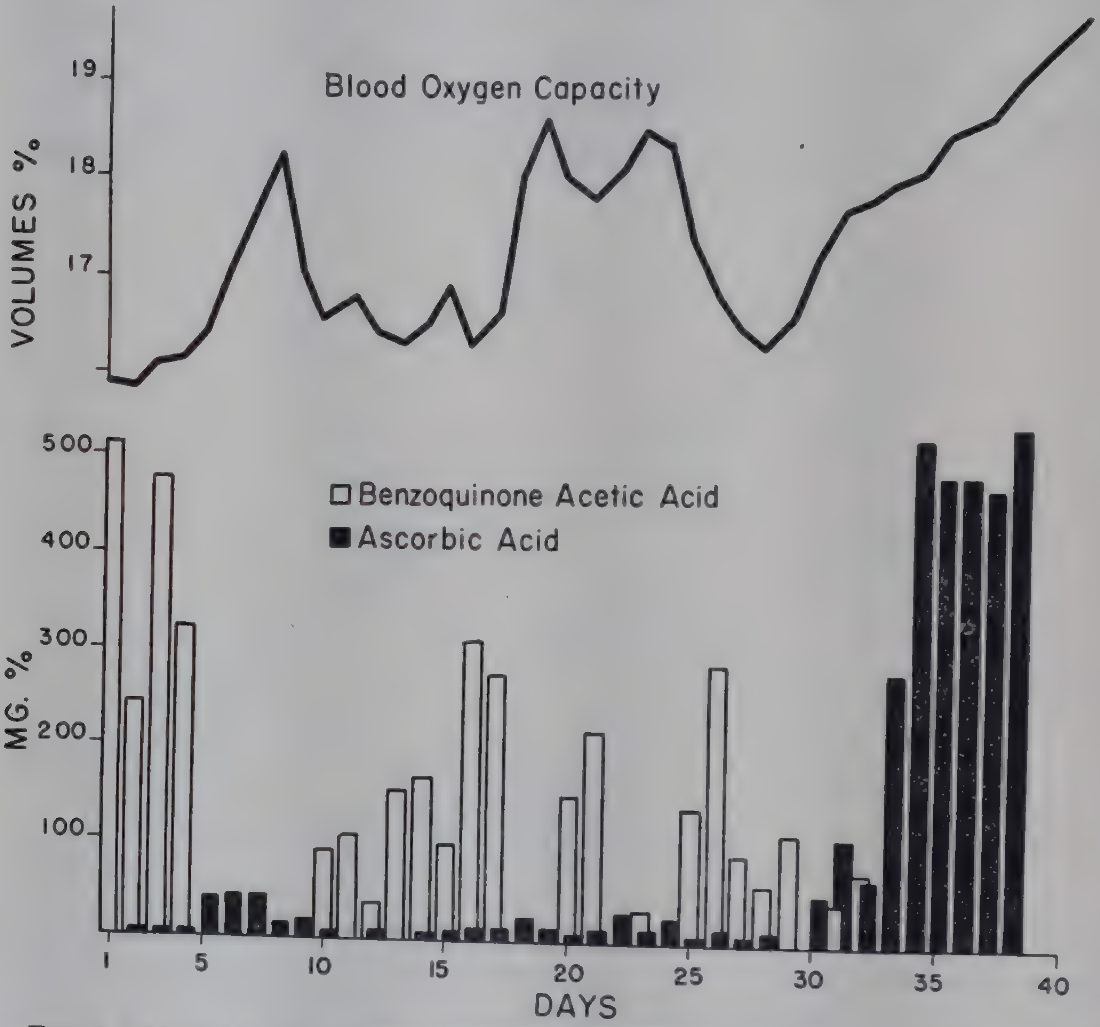

FIG. 3. Changes in the urinary excretion of benzoquinoneacetic acid and ascorbic acid and the oxygen capacity of the blood in autoxic enterogenous cyanosis.

no quinone could be found. From the 30th day onward the subject was given 500 mg. of ascorbic acid by mouth and 500 mg. intravenously. There was rapid cessation of the excretion of benzoquinoneacetic acid, the urinary excretion of ascorbic acid rose to an average of 457 mg. per 24 hours, the oxygen capacity of the blood rose, the methemoglobin band paled and finally almost disappeared on spectroscopic examination, and the cyanosis became less apparent and finally was not visible.

SUMMARY

A substance capable of methemoglobin formation *in vitro*, giving the chemical reactions of a *p*-quinone, was obtained from the urine of subjects showing decreased urinary excretion of vitamin C, but only during those periods when the urinary excretion of vitamin C was diminished. This was identified as benzoquinoneacetic acid on the basis of its chemical reactions, including the preparation of a derivative, the dimethyl ether of homogentisic acid, and the determination of its oxidation-reduction potential, that of homogentisic acid. The assumption was made that it was an obligate intermediate in the normal catabolism of tyrosine and phenylalanine, and its existence was so short as to be undemonstrable chemically except for the appearance of a characteristic absorption band in the ultraviolet region on spectrophotometric examination of the blood serum and urine. This is similar to many other physiological mechanisms which function imperceptibly under normal conditions but become more apparent through exaggerations inherent in their pathological manifestations. In rheumatic fever and scurvy this quinone persists for a longer time, owing to interruption of the normal tyrosine catabolism at the quinoid stage as a sequel to lack of sufficient available ascorbic acid, a key component of the enzyme system necessary for complete tyrosine oxidation. Here the benzoquinoneacetic acid may be determined chemically in the urine. In enterogenous cyanosis the quinone is produced in such quantities that a large part of the hemoglobin of the blood stream is converted into methemoglobin.

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THE MECHANISM OF RETINAL VITAMIN A FORMATION*

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Rhodopsin, the photosensitive pigment of night vision in most vertebrates, is a complex lipoprotein which can be extracted from the retinal rod cells by mild detergents such as aqueous digitonin. Dissolved rhodopsin is characterized by an absorption maximum at about 500 mμ in the blue-green region of the spectrum. On exposure to light it bleaches with the release of yellow lipides. The bleaching process is complex, involving a primary photoprocess (1, 2) and secondary dark reactions. At temperatures near 0° the rate of the dark reactions is greatly slowed and an unstable photoproduct, transient orange (3, 4), can be detected. This substance has a maximum spectral absorption between 465 and 480 mμ in aqueous digitonin. If the solution is warmed to room temperature, transient orange is rapidly converted to the more stable pigment, indicator yellow (5, 6), with a peak at about 445 mμ in acid and 360 mμ in basic solution (7).

If frog retinas are illuminated just long enough to bleach them to a yellow color and are then shaken with petroleum ether, a third substance, retinene (8), can be extracted. It has the properties of a carotenoid, giving a blue color with antimony trichloride in chloroform (9), due to an absorption band with a peak at 664 mμ. It has been suggested that retinene is vitamin A aldehyde (10). The absorption spectra of retinene and other colored lipides of the rhodopsin series are shown in Fig. 1.

When retinas are illuminated for a long time, their yellow color fades, and shaking with petroleum ether yields a substance with the properties of vitamin A. Wald has incorporated retinene as a precursor of vitamin A in his well known visual cycle, but the rôle of transient orange and indicator yellow has remained a moot point. Another problem that has been noted by several investigators is the relatively small production of retinene and vitamin A from dissolved rhodopsin, in striking contrast to the behavior of retinal rhodopsin. These discrepancies pointed to the need for new experiments (11) which have led to a more detailed picture of the dark processes following the illumination of rhodopsin. A diagram of these processes is presented as an aid in following the subsequent description of the experiments on which it is based.

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EXPERIMENTAL

Methods

The following procedures were employed in most of the experiments referred to in later sections of this paper. Deviations from this general procedure will be specified in the descriptions of the individual experiments. In all the experimental series the methods were standardized with regard to times, temperatures, stirring, illumination, and drainage losses to minimize individual variation.

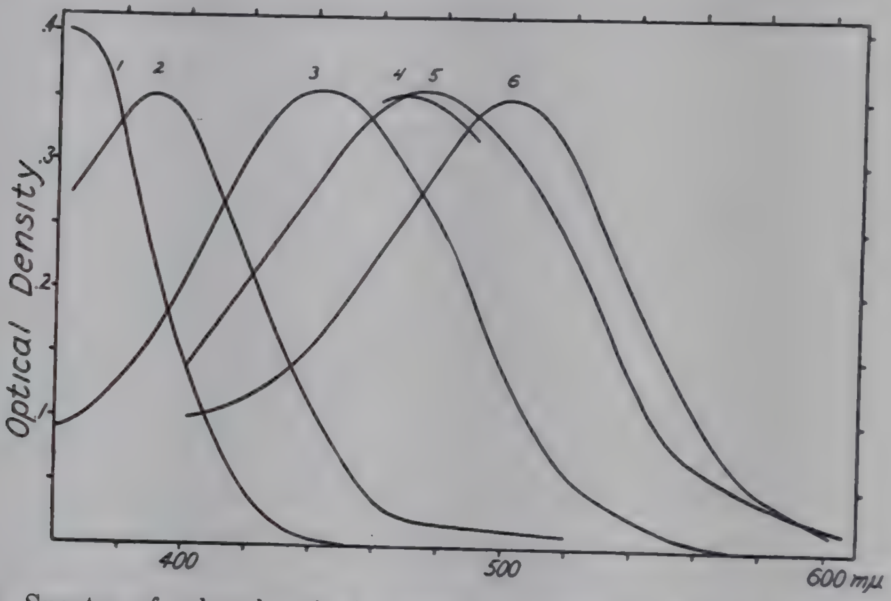
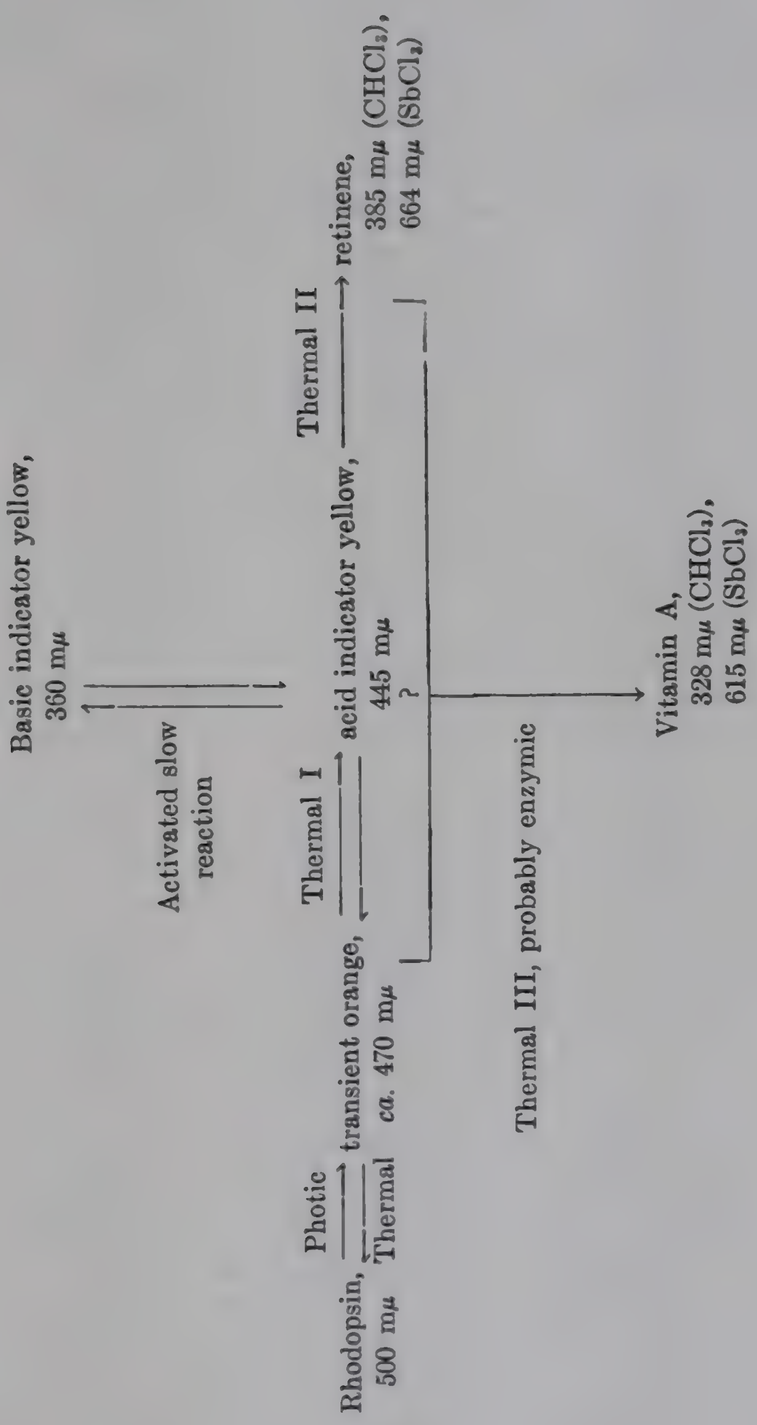


FIG. 1. Spectra of colored retinal pigments. Curve 1, alkaline indicator yellow in alcohol, from retinas pretreated with sodium carbonate; Curve 2, retinene in chloroform; Curve 3, acid indicator yellow in alcohol; Curve 4, aqueous transient orange, from Lythgoe and Quilliam; Curve 5, acid indicator yellow in chloroform; Curve 6, rhodopsin in aqueous digitonin.

The visual pigments were obtained from the common frog, *Rana pipiens*. After dark adaptation for 2 or more hours at 25°, two or three frogs were decapitated in dim red light and their retinas dissected from the melanotic pigment epithelium in Ringer's solution. The weight of the fresh retinas, minus drainage fluid, was determined on a glass plate, and usually ranged from 100 to 200 mg.

In certain experiments the weighed retinas were hardened for 1 hour in 4 per cent alum (potassium aluminum sulfate), followed by a 1 hour water wash. Since this treatment apparently renders insoluble nearly all the proteins of the retina except rhodopsin, it was not employed when components other than rhodopsin were desired in the subsequent extract.

The retinas were placed in a 12 ml. conical centrifuge tube and extracted


Rhodopsin, 500 mμ
Photic
Thermal
transient orange, ca. 470 mμ
Thermal I
acid indicator yellow, 445 mμ
Thermal II
retinene, 385 mμ (CHCl₃), 664 mμ (SbCl₃)
Activated slow reaction
Basic indicator yellow, 360 mμ
?
Thermal III, probably enzymic
Vitamin A, 328 mμ (CHCl₃), 615 mμ (SbCl₃)

by grinding with a glass rod for 1 minute in 0.2 ml. of 3 per cent aqueous digitonin. After 10 minutes and 20 minutes at 25°, the suspension was again ground, and then was spun in a Sorvall angle centrifuge at a high speed for 10 minutes. The residue was reextracted for 10 minutes with 0.2 ml. of water. 0.2 ml. of phosphate-citrate buffer at a pH appropriate to the particular experiment was added to the pooled first and second extract, and the resulting pH determined by a Beckman 1 drop glass electrode.

The digitonin solutions of rhodopsin were bleached 30 seconds in light from a 100 watt bulb at a distance of 3 inches. A half inch of water and a Corning No. 3389 (yellow) filter were placed in the beam to reduce possible side effects of infra-red and ultraviolet light. The progress of the thermal reactions was followed at 25° in light from a 200 watt bulb 10 feet distant, to prevent regeneration of the bleached rhodopsin.

The carotenoid fraction was extracted from the bleached rhodopsin solutions by shaking the solutions directly with 10 volumes of petroleum ether, or by adding 9 volumes of absolute methanol to the solution and shaking for a few seconds with two 5 ml. portions of petroleum ether. Since the latter procedure led to prompt quenching of enzymic activity and quicker separation of the layers after shaking, it was used except where otherwise indicated. The petroleum ether was then evaporated to dryness in a 75 ml. test-tube at room temperature *in vacuo*. 1.5 ml. of 5 per cent alcoholic KOH were added to the residue and allowed to stand 30 minutes in the dark at 25°, a step which decreased the incidence of turbidity in the subsequent $SbCl_3$ test. After addition of an equal amount of water the alkaline alcohol solution was shaken with two 5 ml. portions of petroleum ether. The residue from the latter was transferred to the 1 cm. optical cell with 0.35 ml. of chloroform. After the cell was placed in the photoelectric spectrophotometer (12), 0.65 ml. of antimony trichloride in saturated chloroform solution at 25° was mixed with a drop of acetic anhydride to remove moisture. This solution was then added to the sample in the optical cell. The 35:65 ratio of the sample to the reagent was chosen in preference to the 1:10 ratio usually employed in the Carr-Price test because it permitted more accurate transfer of small samples, and led to readings that were not significantly different from those obtained by the standard method.

Within 15 seconds after the addition of the antimony trichloride the galvanometer deflection of the spectrophotometer was read. Wave-lengths were changed with the aid of a notched wave-length drum and spring catch to insure maximum rapidity in recording the fleeting blue color. Since the circuit response was linear, the readings could be converted to optical density ($\log I_0/I$), which was finally corrected for the density of the cell and solvent. The observed densities were corrected, by extrapolation of the time-density curve, for the loss in density after addition of the reagent and before the readings.

Correction was made, when necessary, for the mutual interference of the vitamin A and retinene absorption bands in the Carr-Price test. This correction is necessary when appreciable quantities of both carotenoids are present, since the density of the antimony trichloride complex of vitamin A at 664 mμ is about 0.2 of that at the vitamin A maximum, 615 mμ. Likewise the density of the antimony trichloride complex of retinene at 615 mμ is about 0.7 of that at the retinene maximum, 664 mμ. The portion of the observed density at 615 mμ which is due to vitamin A may be represented by x. Similarly the portion of the density at 664 mμ which is due to retinene may be called y. Then the observed density at 615 mμ $= (x + 0.7\, y)$, and the observed density at 664 mμ $= (y + 0.2\, x)$. These two equations may then be solved for x and y.

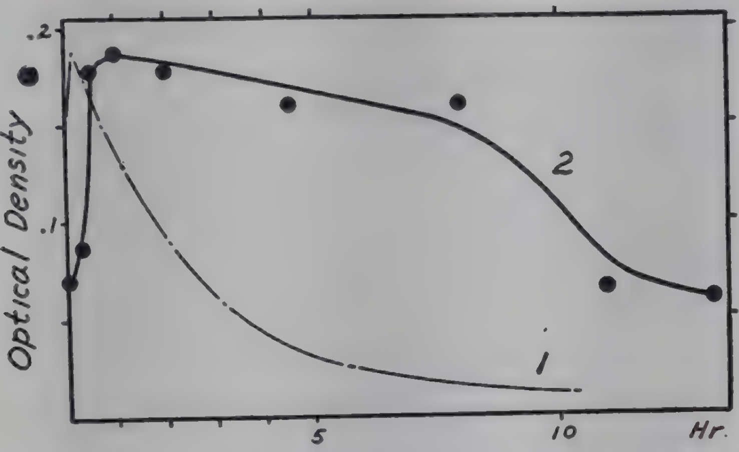


Fig. 2. Thermal changes in bleached rhodopsin solutions at pH 3.9. Curve 1 density at 470 mμ of transient orange and acid indicator yellow from 164 mg., wet, weight of retinas, in 1.6 ml. of aqueous digitonin at 25°. Curve 2, density of $SbCl_3$ test for retinene per 100 mg., wet weight.

Retinene Production in Rhodopsin Solutions—Rhodopsin solutions were prepared from alum-soaked retinas, as described in "Methods." In the first series the buffer was omitted to obtain an acid extract at pH 3.9, the volume of which was brought to 1.5 ml. with water. During the first 8 minutes following bleaching at 25° the initial photoproduct, transient orange, was replaced by the intensely yellow pigment, acid indicator yellow. This substance then bleached gradually over the course of 10 hours. These thermal changes were recorded at 470 mμ, at which wave-length the absorption of aqueous retinene is nearly zero, and are shown in Fig. 2, Curve 1.

The amount of retinene present at various times following bleaching of acid solutions of rhodopsin was then determined as follows: 9 volumes of methanol were added to each solution tested. An equal volume of water was then added, and the resulting 50 per cent methanol solution was shaken with three 5 ml. portions of petroleum ether. The dried residue from the petroleum ether was then saponified and tested by the Carr-Price reaction as described in "Methods." The results are given in Fig. 2, Curve 2 Each point on Curve 2 represents the density at 664 mμ due to retinene extracted from a separate 0.4 ml. bleached solution which had been allowed to stand for the indicated time at 25°. It can be seen that transient orange released no significant amount of retinene, whereas acid indicator yellow formed retinene until the acid indicator yellow had completely disappeared. Although aqueous transient orange was rapidly converted to the retinene precursor, acid indicator yellow, the conversion rate in 50 per cent methanol was apparently slow enough so that little retinene was formed in the 15 minutes required for extraction of the carotenoid fraction from the 50 per cent methanol.

It is also clear that the retinene itself was labile, so that only a part of the total potential retinene was present at any time. The total retinene was determined by shaking a bleached solution of acidic rhodopsin with 10 volume portions of petroleum ether for 5 minute periods at intervals of about an hour. The emulsions were separated by centrifugation, and the residues from the petroleum ether were brought without saponification into chloroform. They were then tested with antimony trichloride, as already described. The retinene value for each petroleum ether extract was added to the cumulative value for the previous shakings, giving a total retinene production of 0.4 density units at 664 mμ in 9 hours following bleaching, a considerably higher value than the maximum, 0.24, obtained by a comparable single determination based on extraction of the retinene from 90 per cent methanol.

Retinene production was strongly influenced by pH, both in rate and amount. A solution of rhodopsin from alum-pretreated retinas, brought to pH 6.7, exhibited an induction period of retinene formation only 2 minutes in length, much shorter than the 8 minutes required at pH 3.9. Very little retinene could be obtained from solutions above pH 7. The antimony trichloride test yielded a faint blue color, which was probably due to preformed vitamin A, traces of retinene, and possibly a faint reaction of indicator yellow or transient orange. In alkaline solution the retinene precursor, acid indicator yellow, was reversibly converted to its stable basic form.

Vitamin A Production in Rhodopsin Solutions—Wald has shown that retinene is an intermediate in the formation of vitamin A by the bleached retina. When retinas are soaked in alum before bleaching, vitamin A is no longer formed, even though the pH is brought back to neutrality. Similarly digitonin solutions of rhodopsin from retinas so treated yield retinene alone. In fact, it has been emphatically denied by several workers that the formation of vitamin A in rhodopsin solutions has ever been observed.

However, Wald (13) has shown that fresh neutral solutions of rhodopsin exhibit a thermal fading after illumination similar to that of fresh retinas in the course of vitamin A formation. This clue made it necessary to reinvestigate the production of carotenoids by rhodopsin solutions.

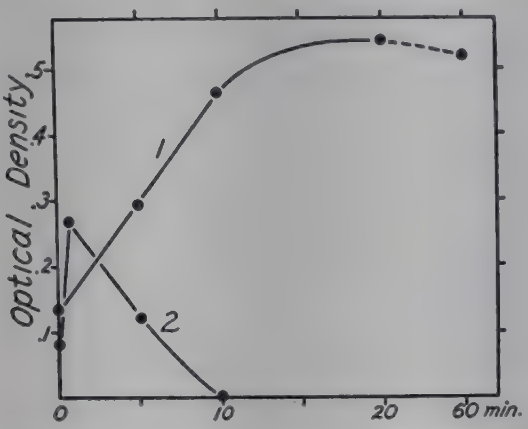


FIG. 3. Vitamin A formation by unbuffered extract per 100 mg., wet weight, at 25° Curve 1, vitamin A; Curve 2, retinene.

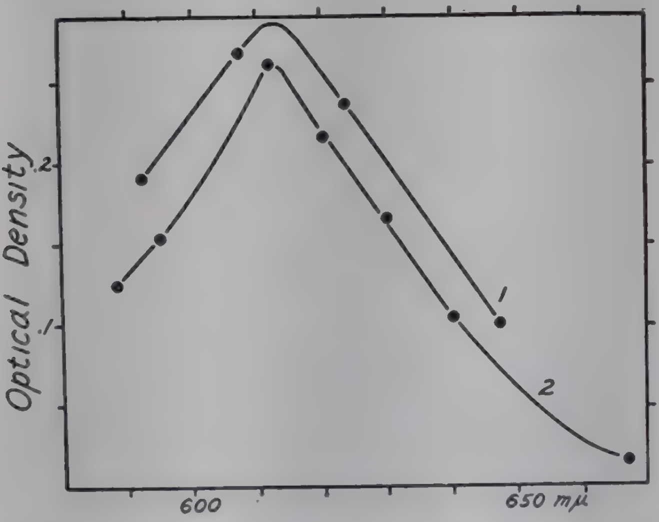


FIG. 4. Absorption spectra of $SbCl_3$ test per 100 mg., wet weight, 1 hour after bleaching. Curve 1, petroleum ether extract of whole retinas; Curve 2, petroleum ether extract of aqueous digitonin solution of rhodopsin bleaching products.

A fresh, unbuffered solution of rhodopsin was bleached for 30 seconds in the light described above, and was then kept at a distance of 10 feet from a 200 watt lamp to prevent regeneration of the rhodopsin. Aliquots were treated with alcohol at various times after bleaching at 25° and stored over solid carbon dioxide until they could be tested for vitamin A and retinene. The course of this experiment is shown in Fig. 3, and the absorption spectrum of the final blue color is shown in Fig. 4. Figs. 3 and 4 show that fresh extracts of rhodopsin formed vitamin A as efficiently as did whole retinas. When an unbleached extract of fresh retinas was added to a similar extract of alum-treated retinas, the amount of vitamin A formed was the same as that of two fresh extracts. The numerical data of these experiments are given in Table I, and show that vitamin A formation was induced by an independent, alum-labile factor. The factor could be obtained free from rhodopsin by bleaching retinas until they had formed their maximum amount of vitamin A. They were then frozen and dried *in vacuo*. The dried retinas were washed on a glass filter for 1 hour with petroleum ether at 5°, and then extracted with digitonin as usual. Such extracts yielded no trace of vitamin A, but induced the formation of maximum amounts of vitamin A from alum-pretreated bleached rhodopsin solutions at pH 6.7.

Effect of Trypsin on Rhodopsin and on Vitamin A Formation—It seemed probable that the activating factor was an enzyme or an enzymic system. If this were the case, it might be expected that its activity would be destroyed by trypsin, to which rhodopsin has been reported insensitive (14). A 10 per cent suspension of Pfanstiehl "trypsin powder (1:110)" was dialyzed 6 hours in a refrigerator and then centrifuged at a high speed for 10 minutes in an angle centrifuge. An equal volume of the supernatant was added to one-half of a digitonin solution prepared as follows: twenty freshly excised frog retinas were extracted for 20 minutes at 26° in 0.3 ml. of 3 per cent aqueous digitonin, and similarly reextracted 10 minutes in 0.2 ml. of digitonin. The pooled rhodopsin and trypsin solution was brought to pH 7.0 by 1 ml. of phosphate-citrate buffer of pH 7.1.

TABLE I

Induction of Vitamin A Formation by Fresh Extracts of Rhodopsin

Density of $SbCl_3$ color test per 100 mg., wet weight, pH 7.5, 1 hour after bleaching, 27 ± 2°. The data in the first column give the range of five experiments; the other columns, the average of duplicate experiments.

Wave-length	Fresh solutions	Alum-pretreated solutions	Fresh + alum-pretreated
mμ			
615	0.25 ± 0.06	0.065	0.25
664	0.06 ± 0.02	0.085	0.05

An equal amount of the buffer was added to the other half of the rhodopsin solution as a control. The trypsin-treated solution was allowed to stand for 1 hour at 25° in the dark before testing. The rhodopsin content was determined as density fall at 500 mμ following 30 seconds bleaching by a 100 watt lamp at 3 inches. In two such experiments the rhodopsin values of the controls were 0.18 and 0.15, while the corresponding values in the trypsin solutions were 0.21 and 0.18. Thus the trypsin treatment had no significant effect on the rhodopsin. However, trypsin-treated solutions formed no vitamin A, showing that the activating factor was probably a protein. The fact that rhodopsin, which is also a protein, was not similarly destroyed may be due to its large lipide prosthetic group.

Aging of Vitamin A Factor—Aqueous digitonin extracts of fresh retinas were prepared as quickly as possible, according to "Methods." In this series the buffer was omitted and the pH was thus maintained by the extracted tissue buffers at about 6. Each extract was kept in the dark at 25° for a definite time and then bleached 30 seconds. The bleached solution was then kept in light for 10 minutes at 25.0°. The reaction was terminated by the addition of 9 volumes of methanol, from which the carotenoids were extracted as described in "Methods." The results are shown in Fig. 5, in which each point represents the average of two separate preparations. It can be seen that the rate of vitamin A formation, measured at 615 mμ, remained high for about 2 hours following the start of the extraction, and then fell rapidly to the level of preformed vitamin A. Simultaneously the retinene curve, measured at 664 mμ, rose. These curves suggest that the rhodopsin solutions contained a labile factor originally present in excess, but which in 2 hours had deteriorated to a low enough concentration to limit the rate of vitamin A formation and cause an accumulation of retinene.

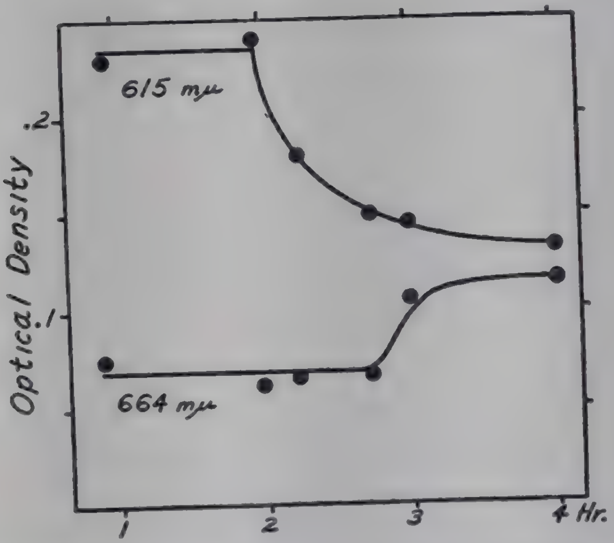


FIG. 5. Aging of vitamin A factor in unbuffered solutions. Ordinate, density of $SbCl_3$ test per 100 mg., wet weight. Abscissa, time from start of digitonin extraction. Each point gives the average of duplicate experiments.

Effect of pH on Vitamin A Formation—Buffered digitonin solutions of rhodopsin from freshly excised retinas were prepared as described in

"Methods." After determination of the pH each solution was illuminated and allowed to stand for 10 minutes at 25.0°. The action was stopped with 9 volumes of methanol, and the carotenoid production was measured at 615 and 664 mμ as before. The vitamin A formation was plotted against pH and is shown in Fig. 6. In this curve each point represents the vitamin A extracted from a separate, bleached preparation. The values have been corrected for the density due to preformed vitamin A, 0.02 per 100 mg., which was determined by shaking an aqueous digitonin solution of rhodopsin at pH 6.7 for a total of 15 minutes in the dark with two 5 ml. portions of petroleum ether. The activity of the vitamin A factor is seen to be maximum at pH 6.7 and falls to zero at about pH 5.5 and 7.9. The shoulder on the acid side coincides with the appearance of turbidity on addition of the acid buffer.

Precursor of Vitamin A—A solution of bleached acidic rhodopsin of the type shown in Fig. 2, Curve 2, gradually forms retinene from acid indicator yellow. Such a solution was allowed to stand for 4 hours after bleaching at 25°, by which time about three-quarters of the indicator yellow had been converted to retinene. The solution was then brought to pH 6 and mixed with an equal volume of fresh rhodopsin solution containing the vitamin A factor. After standing for 1 hour at 25° the bleached mixture was tested for carotenoids as usual. The retinene was found to have been completely removed, and the vitamin A density, 0.27 per 100 mg. of wet weight, was about that usually obtained from freshly prepared solutions at this pH. It therefore seems that the vitamin A factor can act on retinene.

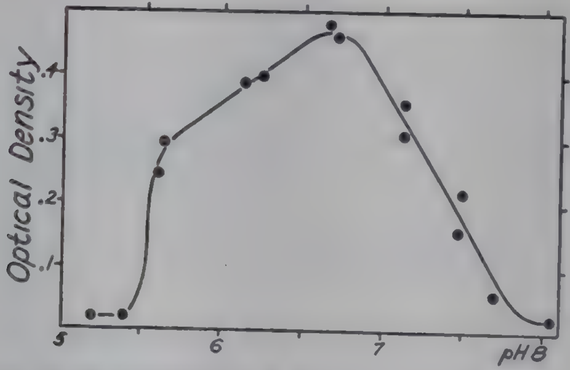


FIG. 6. Effect of pH on vitamin A formation per 100 mg., wet weight

It is, however, equally clear that indicator yellow can also be converted to vitamin A in freshly bleached solutions. This raises the question of whether the conversion is direct or via retinene. If retinene is an intermediate, two possible modes of action of the vitamin A factor present themselves. In the first type, the vitamin A factor can be pictured as having a dual nature, catalyzing the transformation of acid indicator yellow into retinene, and then converting retinene into vitamin A. The second possibility is that the accumulation of retinene inhibits the further conversion of acid indicator yellow to retinene, and that the vitamin A factor operates by removing the inhibition through the conversion of retinene to vitamin A.

If the vitamin A factor catalyzes the formation of retinene from indicator yellow, one might expect a transitory increase in retinene formation immediately after bleaching a vitamin A-active solution, compared with the retinene formation in an inactive solution. An active solution at pH 6.7, prepared according to "Methods," was allowed to stand at 25° for 75 seconds after the start of bleaching. It was then treated with 9 volumes of methanol and analyzed for carotenoids. Vitamin A and retinene densities of 0.14 and 0.05 per 100 mg. of wet weight were found. An inactive rhodopsin solution at the same pH and time after bleaching gave a corresponding retinene value of 0.08. Thus there is no present evidence that vitamin A formation is accompanied by catalysis of retinene formation.

The second possibility, that removal of retinene accelerates indicator yellow fading, has also been briefly investigated. A bleached acidic solution of rhodopsin at pH 3.9 was shaken violently for 20 minutes at 27° with 10 volumes of petroleum ether. The resulting density of the aqueous indicator yellow at 470 mμ was found to be 10 per cent lower than the control, a similar solution shaken with nitrogen. This difference is of dubious significance and does not support the view that the bleaching of indicator yellow is influenced by the removal of retinene.

If we assume that indicator yellow is a precursor of vitamin A, the question arises as to whether the acid or basic form is the essential component. This is a difficult problem because of the probable existence of these two forms in an equilibrium mixture controlled by the pH. The fall in rate of vitamin A formation with rise in basicity toward pH 8 suggests that the acid form is the actual precursor of vitamin A.

DISCUSSION

The primary photoproduct of rhodopsin, transient orange, was considered by Lythgoe (5) to be the precursor of indicator yellow. The absorption spectrum of transient orange closely resembles that of the acid form of indicator yellow, as may be seen in Fig. 1. Lythgoe and Quilliam (3) erroneously concluded that transient orange at 3° is distinguishable from indicator yellow by the failure of the former to act as a pH indicator. Actually acid indicator yellow at the same temperature proved to be similarly unresponsive to the addition of a neutral buffer, as may be seen in Fig. 7. The sluggish response of indicator yellow, like that of certain laboratory indicators (3), is probably an example of a secondary acid neutralization. This may lead to an inhibitory effect of cooling such as is found in the case of 4,4′,4″-trinitrophenylmethane in alkaline alcohol at −30° to −80°. When this solution is acidified, the blue color of the triphenylmethide ion fades more and more slowly the lower the temperature, showing that a typical activated slow reaction is taking place; *viz.*, the transformation of the blue secondary base into a primary base (15). The neutralization of a primary base requires no energy of activation and therefore proceeds rapidly even at low temperatures. Although response to pH does not aid in distinguishing transient orange from acid indicator yellow, they are apparently different, since transient orange, unlike acid indicator yellow, does not release retinene.

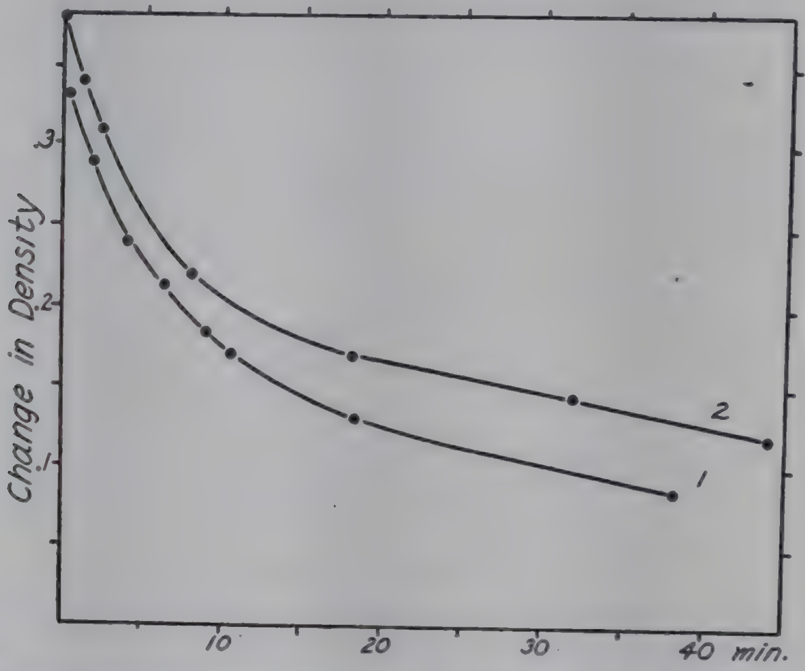


FIG. 7. Curve 1, decomposition of transient orange at pH 7.13 and 3°, from Lythgoe and Quilliam; Curve 2, response at 450 mμ of alum-pretreated, bleached digitonin extract to the change of pH from 5 to 7, at 3°; actual densities are 0.1 of the scale at the left.

The events in bleaching rhodopsin solutions can be correlated with the following three thermal processes distinguished by Wald (13): Thermal Component I, the absorption rises at 440 mμ in solutions below pH 7; Thermal Component II, the absorption falls at 480 mμ and rises below 435 mμ, in solutions below pH 9; at a lower pH the maximum of bleaching and the node shift toward shorter wave-lengths; Thermal Component III, in fresh neutral solutions the absorption decreases at all wave-lengths.

The interpretation of these processes in the light of present evidence ollows: Thermal Component I, acid indicator yellow is formed from transient orange; Thermal Component II, transient orange forms indicator yellow; at a lower pH acid indicator yellow is formed and bleaches to retinene; Thermal Component III, in fresh neutral solution colorless vitamin A is formed.

Little is known concerning the chemistry of these changes. Krause (4, 6, 7) has performed some interesting analyses on the visual lipides derived from bovine retinas, but, since he does not state that the animals had been previously dark-adapted, no conclusions can be drawn concerning the position of the lipides in the visual cycle. The formation of retinene apparently is not an enzymic process, since it has been observed in chloroform solutions of its precursor (16). Chase and Hagan (17) have shown that the absence of oxygen is without effect on the spectrophotometric course of rhodopsin bleaching.

The retinal factor which induces the formation of vitamin A exhibits the pH dependence, lability, and protein nature characteristic of enzymes. It is interesting, in this connection, to note that the conversion of synthetic retinene to vitamin A by a reductase in rat liver and intestinal mucosa has recently been reported (18). However, until evidence is offered in support of these sites of conversion, it can be equally well assumed that the actual formation of vitamin A took place in the retina.

SUMMARY

1. The primary photoproduct of rhodopsin, transient orange, is converted in a few minutes at 25° to a more stable lipide, indicator yellow, which is deep yellow in acid and almost colorless in basic solution. The change from the acid to the basic form is reversible, but very sluggish near 0°.

2. Acid indicator yellow in aqueous digitonin is converted in a few hours to the carotenoid, retinene.

3. Fresh bleached solutions of rhodopsin form large quantities of vitamin A.

4. Vitamin A formation is induced by a labile, probably enzymic factor active between pH 5.5 and 7.9, with maximum activity at pH 6.7.

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BLOOD SUGAR AFTER INJECTION OF ACETOACETATE

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A number of investigators (1) have attempted to explain the decreased utilization of carbohydrate in conditions accompanying food intakes low in carbohydrate and high in fat. In the case of the formation of increased amounts of ketone bodies due to such conditions or to diabetes, Nath and Brahmachari (2) have attributed at least a part of the decreased utilization of carbohydrate to an inactivation of available insulin by these intermediary fat metabolism products. "Insulin-refractory" cases were explained in this way. They also reported (3) that repeated injections of acetoacetic, β-hydroxybutyric, and pyruvic acids caused hyperglycemia in rabbits, with fasting values increasing from day to day after daily injections.

We had previously noted (4, 5) that rats which had been on a low protein-high fat diet were much more susceptible to ketosis when fasted and that they also exhibited a decreased glucose tolerance. In view of the above findings, it seemed desirable to obtain additional information regarding the effect of the ketone bodies on carbohydrate metabolism in such animals. When rats instead of rabbits were used as the experimental animal, we failed to confirm the findings of Nath and Brahmachari. Instead of elevated blood sugar levels, hypoglycemia developed after the injection of very large amounts of acetoacetate.

EXPERIMENTAL

In the first experiment, female white rats averaging 250 gm. in weight were used to test the hyperglycemic effect of the injection of ketone bodies. Females were selected, since it is possible that ketosis may produce a more marked effect on their carbohydrate metabolism than on that of males (6). All animals of this study were fed a diet similar in composition to that previously used (4), with 25 per cent casein and 15 per cent Crisco. The food was available for 24 hours to a part of the rats, to others for 7 hours, and to still others only 3 hours a day during the preliminary period of 2 weeks for establishing new feeding habits. The animals on each of these feeding régimes were divided into three groups. For blood sugar determinations, 0.2 cc. of blood was milked from the clipped tails of all these animals after 18 hours fasting and at intervals of 1, 2, and 3 hours thereafter. After the fasting blood sample was taken, the first group was injected intraperitoneally with 1 cc. of 1 per cent saline per 100 gm. of body weight the 1st day, and then with 18 mg. of acetoacetic acid, in 1 cc. of saline solution adjusted to pH 7.3, per 100 gm. of body weight for the next 3 days. The second group received only the saline on all 4 days and the third group was not injected. Four samples of blood for sugar determinations were taken from each animal. All saline injections contained amounts of sodium equivalent to that in the acetoacetic acid.

A second experiment was undertaken to study the effect of injected acetoacetate upon endogenous insulin available for sugar utilization during glucose tolerance tests. Female rats averaging 220 gm. were used for the tolerance tests. Control values were obtained 1 week with glucose in saline and then with glucose in acetoacetic acid the following week. The first four animals received intraperitoneally 3.5 gm. of glucose per kilo in 1 per cent saline, followed by 18 mg. of acetoacetic acid and the same amount of glucose per 100 gm. of body weight. The second group was treated in like manner, except that less glucose, 2.0 gm. per kilo, and twice as much acetoacetic acid were given them.

A study to determine the effect upon the insulin-secreting mechanism of daily injections of acetoacetate in increasing amounts for an extended period was made in a third experiment. The same diet was fed to male rats averaging 230 gm. in weight. Blood sugar determinations were made as controls on the fasting levels and at 1 hour after the subcutaneous injection of 1 per cent saline at the start of the experiment. Similar determinations were made once each week, before and after the subcutaneous injection of acetoacetic acid or saline daily for 4 weeks. The initial injection of 18 mg. of acetoacetic acid per 100 gm. of body weight was doubled each week up to 144 mg. the last week. The controls received saline containing equivalent amounts of sodium. For comparison, the latter amount of acetoacetic acid or saline was injected for only 1 day in other animals (approximately 300 gm.) and blood sugar determined during fasting and 1 hour after injection.

All blood sugars were determined in triplicate by the method described by Nelson (7). The acetoacetic acid injected was prepared by hydrolyzing ethyl acetoacetate with sodium hydroxide in the cold and adjusting finally to pH 7.3 with hydrochloric acid. Urine was collected for 6 hours and then the following 18 hours after all acetoacetate injections, and qualitative tests for ketone bodies were made by the nitroprusside method. The room temperature during these experiments was maintained at 26° ± 1°.

Apparent differences in results were tested for significance by the t method of Fisher (8), and only those having a P value of 0.01 or less were considered significant.

Results

The various feeding régimes of the preliminary period in the first experiment did not cause any apparent differences in the blood sugar levels of the nimals which had been divided into the three groups. Hence the data on ll of the animals in each of these groups have been pooled. In all cases the initial concentration of the fasting blood sugar on the 3rd day after the acetoacetic acid injections were begun was significantly higher than the control values of the 1st day, as previously reported (3) for rabbits. This was also true for the average values, shown in Table I, on rats whether they received the keto acid, saline only, or no injections whatsoever.

The acetoacetic acid injections did not significantly affect the blood sugar levels during the 3 hours following injection, although ketonuria of varying degrees always ensued. The injection of 18 mg. of acetoacetic acid per 100 gm. of body weight was used because it did produce a definite ketonuria and, on a weight basis, was about twice as much as Nath and Brahmachari gave to their rabbits (2).

TABLE I

Effect of Injection of Keto Acid and Treatment upon Blood Sugar Levels

The animals were fasted 18 hours. They received the diet *ad libitum* during the remaining 3 hours after collection of blood samples each day. 1 cc. of 1 per cent saline or 18 mg. of acetoacetic acid per 100 gm. of body weight were injected after collection of the fasting blood sample.

Day	No. of rats	Injection	Blood sugar*			
			Fasting	1 hr.	2 hrs.	3 hrs.
			mg.	mg.	mg.	mg.
1	10	Saline	78 ± 2	79 ± 1	80 ± 3	73 ± 2
	8	"	80 ± 2	82 ± 3	82 ± 3	81 ± 3
	4	None	82 ± 5	85 ± 5	85 ± 5	78 ± 3
2	10	Keto acid	86 ± 3	89 ± 1	89 ± 2	85 ± 2
	8	Saline	88 ± 4	91 ± 4	88 ± 5	87 ± 4
	4	None	93 ± 5	100 ± 4	102 ± 3	95 ± 4
3	10	Keto acid	92 ± 1	92 ± 2	95 ± 3	92 ± 3
	8	Saline	97 ± 2	95 ± 3	95 ± 3	92 ± 3
	4	None	97 ± 3	93 ± 9	91 ± 3	90 ± 1
4	10	Keto acid	96 ± 2	94 ± 3	93 ± 2	93 ± 2
	8	Saline	100 ± 2	102 ± 3	97 ± 5	96 ± 4
	4	None	100 ± 5	94 ± 10	95 ± 2	95 ± 4

* ± the standard error.

In the second experiment, the glucose tolerance was not significantly changed from the control values by the simultaneous injection of glucose and acetoacetic acid. A loss of some sugar in the urine and no appreciable change in the tolerance curves in the first animals suggested the use of less glucose and more acetoacetic acid. Both tests, summarized in Table II, gave similar results. There was no indication that the injected ketone body had affected the endogenous insulin available for sugar utilization during these tests.

The results of the last experiment are given in Table III. The daily injection of from 18 to 70 mg. of acetoacetic acid per 100 gm. of body weight during a 3 week period did not significantly change the blood sugar during fasting or 1 hour after injection. Subcutaneous injection was used to prolong the period of absorption. After the injection of 144 mg. of the keto acid each day during the 4th week, a definite hypoglycemia did develop 1

Table II

Effect of Keto Acid Injection on Glucose Tolerance

The four animals in each group were fasted 16 hours. The glucose was dissolved in saline so that the amount injected would contain sodium equivalent to that in the acetoacetic acid solution (pH 7.3).

Injection		Blood Sugar*					
Glucose	Aceto-acetic acid	Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	5 hrs.
gm. per kg.	*mg. per 100 gm. rat*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
3.5	0	75 ± 4	318 ± 20	226 ± 27	136 ± 6	127 ± 4	109 ± 7
3.5	18	75 ± 10	288 ± 10	176 ± 21	136 ± 13	128 ± 10	103 ± 5
2.0	0	72 ± 2	161 ± 17	119 ± 4	107 ± 7	103 ± 7	88 ± 4
2.0	36	78 ± 3	169 ± 12	117 ± 6	105 ± 3	106 ± 6	94 ± 7

* ± the standard error.

Table III

Blood Sugar after Daily Injection of Keto Acid

Control values on four animals were obtained after an 18 hour fast and then 1 hour after the subcutaneous injection of saline. Acetoacetic acid was injected daily for 4 weeks and corresponding sugar determinations were made at the end of each week. A 1 day test was made on ten animals with the keto acid and on ten with saline.

Food intake per day	Change in weight	Wks. injected	Keto acid injected	Blood sugar*	
				Fasting	1 hr. after injection
gm.	*per cent*		*mg. per 100 gm. rat*	*mg. per cent*	*mg. per cent*
		0	0	76 ± 8	80 ± 7
15.5	4.4	1	18	75 ± 2	75 ± 3
15.3	0.8	2	36	71 ± 3	72 ± 2
10.0	0.0	3	72	71 ± 0	68 ± 3
8.0	2.1	4	144	76 ± 1	55 ± 3
		0	0	76 ± 2	75 ± 3
		0	144	80 ± 2	58 ± 4

* ± the standard error.

hour after the injection. However, in another group of animals, a similar hypoglycemia occurred after only one injection of the latter amount of the keto acid. This suggests that the quantity of the acid given was responsible for the lowered blood sugar and not the repeated daily injections. This quantity appears to be between 70 and 140 mg. per 100 gm. when injected subcutaneously into the rat.

DISCUSSION

The effect of the intermediary fat degradation products, the ketone bodies, upon the secreting pancreatic cells and upon the insulin itself would appear to be of considerable importance in carbohydrate metabolism if the views of Nath and Brahmachari are correct. They presented evidence to show that the ketone bodies inactivated insulin, both *in vitro* and *in vivo*, and suggested that the keto acids might first stimulate the pancreatic cells and later cause lesions after fatigue through excessive work.

It seemed possible that the hyperglycemic effect they obtained after repeated injections might be due to the use of a herbivorous animal, or to the short feeding period each day necessary for an 18 hour fast, since a marked change in the intermediary carbohydrate metabolism has been shown to occur after altered dietary habits (9). Also a limited intake of carbohydrate might explain its decreased utilization. In our study neither the injected keto acid nor the short feeding periods can account for the resulting hyperglycemia in the rat. It occurred in the uninjected animal after repeated daily sugar determinations, and normal values were obtained on the 1st day after some time on the 3 hour feeding régime. In contrast, quite similar blood sugar values were obtained when the determinations were made 1 week apart on the animal injected repeatedly (Table III).

The failure of the acetoacetic acid to alter the glucose tolerance and the failure of its repeated injection over a 4 week interval to produce a hyperglycemia do not appear to be compatible with an *in vitro* inactivation of endogenous insulin. However, the hypoglycemia after injection of very large amounts of the keto acid might be explained by a stimulation of the secreting pancreatic tissue. Another interesting possibility is that of a sugar-sparing action of the ketone bodies, associated with decreased glycogenolysis and lowered blood sugar level, since it has been shown that the rate of utilization of the ketone bodies is governed by their concentration in the blood (10) and that they are preferentially used in the presence of usable glucose (11). Also glycogen has been found to be stored in the heart in the presence of large amounts of administered ketone bodies.[1] This study will be continued.

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[1] Lackey, R. W., personal communication.

SUMMARY

A daily hyperglycemia was found in the rat when injected with acetoacetic acid for 3 consecutive days. The elevated blood sugar apparently was not caused by the ketone body, since the same results were obtained with saline or with no injections. Also blood sugar, before and after the administration of smaller amounts of the keto acid which had been given daily for 3 weeks, was normal if blood samples were taken only 1 day each week. The glucose tolerance was also unchanged by simultaneous injection of the ketone body. However, the administration of a large amount of acetoacetic acid (140 mg. per 100 gm.) caused hypoglycemia even after a single injection.

Our data do not support the view that insulin is inactivated by the keto acid. The hypoglycemia, after large amounts of the acetoacetate, is compatible with a stimulation of the secreting pancreatic tissue, or a glucose-sparing action associated with a decreased glycogenolysis and hence a lowered blood sugar level.

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DETERMINATION OF PLUTONIUM IN HUMAN FECES*

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The toxic effects of plutonium on the human body depend on a considerable number of physical and biological factors. One of the most important biological factors is the rate of elimination of plutonium from the body.

The ratio of fecal to urinary excretion of plutonium by the rat is of the order of 10:1. This is, indeed, a favorable ratio. Preliminary information, however, indicates that the excretion ratio for the human is much less favorable.

It is imperative that the fecal excretion of plutonium by the human and the ratio of fecal to urinary output be carefully determined. Information on these factors was extremely limited, partially because of the difficulty of analyzing human feces for extremely small amounts of plutonium.

This report presents a relatively accurate and consistent method of analyzing human feces for trace amounts of plutonium and some of the experiences of this laboratory during the process of developing such a method.

Methods

Collection of Sample—Fecal specimens, representing from 1 to 4 days excretion, are pooled and completely emulsified in 2 M HCl by heating on a steam bath with rapid stirring. The weight of HCl used is about twice that of the feces. A suitable aliquot by weight is taken for analysis. The size of the aliquot chosen depends both on the expected radioactivity and on the total weight of the sample. In general, not more that 250 gm. of the emulsified sample should be used in any one analysis. Two aliquots of each sample are analyzed.

Ashing and Solution of Sample—The aliquot taken for analysis is transferred to a large porcelain crucible and placed in an oven at 110°. When completely dry, after about 24 hours, the sample is heated over a Bunsen burner until a dark gray ash remains. When there is no further evidence of charring and ashing over the flame, the sample is placed in a muffle

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furnace at 800° for 8 to 15 hours. The resulting ash varies in color from white to a reddish white.

Solution of the ash has proved to be a rather difficult problem, and several methods have been tried. The following procedure, however, has proved satisfactory. About 60 ml. of 4 M HCl are added to the crucible containing the ash. The slurry is heated under an infra-red lamp for about 15 minutes. The partially dissolved sample is transferred to a 200 ml. centrifuge bottle and the insoluble portion is centrifuged. The supernatant is transferred to another 200 ml. centrifuge bottle. The insoluble portion is washed once with 4 M HCl, the wash being added to the original solution, and is transferred to a platinum crucible. About 10 ml. of concentrated HF are added, and the slurry is taken to dryness under an infrared lamp. The residue is taken up in about 5 ml. of 4 N HCl. The part still remaining undissolved is centrifuged and discarded. Many unsuccessful attempts were made to find an easy method of dissolving the residue. However, good recoveries have been obtained on feces samples with known amounts of plutonium added at the first step of the ashing procedure and carried through the entire process, in which the insoluble residue is discarded. It is, therefore, thought unnecessary to attempt to dissolve the residue and to include it in the final solution.

Determination of Plutonium in Solutions of Feces Ash—A number of methods for determining plutonium in solutions of feces ash have been tried. These will not be described in detail, since they have all proved inferior to the method finally adopted.

The first method attempted consisted of hydroxide precipitation followed by cupferron extraction. The chloroform phase from the extraction was wet-ashed to destroy the cupferron. Finally LaF_3 precipitation was carried out and the LaF_3 was transferred to a platinum plate and counted in an α-counter. This method was entirely unsatisfactory.

Direct cupferron extractions were carried out on aliquots of ash solutions of various size. About 75 per cent recovery could be obtained from small aliquots. However, the size of the aliquot which could be tolerated seemed to vary from sample to sample and was too small to give adequate sensitivity.

It was found that calcium oxalate precipitation carried reduced plutonium quite successfully. The addition of calcium to the ash solution is not necessary, as the natural calcium content of feces ash is adequate. A description of the method which was finally selected follows.

The ash solution is adjusted to pH 0.4 to 0.7 with methyl violet indicator. 1 ml. of 3 M hydroxylamine hydrochloride is added. The solution is heated under an infra-red lamp for about 2 hours to facilitate the reduction of plutonium. The pH is readjusted with the same indicator. 25 ml. of

0.8 M oxalic acid are added. A drop of ammonium hydroxide is sometimes necessary to start the precipitation. The solution is left standing overnight to insure complete carrying of the plutonium. The precipitate is centrifuged and washed once with 0.1 M oxalic acid. About 10 ml. of fuming HNO_3 are added, and the solution is taken to dryness under an infra-red lamp. The residue is dissolved in 25 ml. of 2 M HCl and transferred to a 125 ml. separatory funnel. 5 drops of 3 M hydroxylamine and 1 mg. of iron are added. The acidity of the solution is adjusted so that a bright green color is obtained with methyl violet indicator. After ½ hour, for reduction of the plutonium, about 1 ml. of 6 per cent cupferron solution is added. Four or five extractions with about 2 ml. of chloroform for each extraction are carried out. More extractions are necessary if all of the iron has not been removed from the aqueous phase. The chloroform phases are collected in a 40 ml. Pyrex centrifuge cone. The cone is placed in a water bath and the chloroform evaporated. 1 ml. of concentrated HNO_3 and 1 ml. of 72 per cent $HClO_4$ are added to the residue. The cone is then placed in an oil bath at about 100°. The temperature of the oil bath is allowed to rise to about 180° over a period of 1 hour. At the end of this time about 1 ml. of a clear or straw-colored $HClO_4$ solution remains. This is diluted to 5 ml. with distilled water. 2 drops of 20 per cent hydroxylamine are added and the solution allowed to stand ½ hour. 200 γ of La^{+++} and 0.5 ml. of 27 M HF are added. The LaF_3 precipitate is centrifuged, transferred to a platinum plate, and counted in an α-counter for 1 hour.

TABLE I

Recovery of Known Amounts of Plutonium from Solutions of Artificial Feces Ash by Calcium Oxalate-Cupferron Procedure*

Added	Recovered	Recovered
counts per min.	*counts per min.*	*per cent*
150	145	97
150	140	93
150	125	83
150	131	87
Mean		90

* A solution whose mineral composition was that which might be expected from a 50 gm. fecal sample.

Results

Recovery by the above procedure has been tested by adding known amounts of plutonium to both artificial and actual solutions of human

TABLE II

Recovery of Known Amounts of Plutonium from Solutions of Human Feces Ash by Calcium Oxalate-Cupferron Procedure*

Added	Recovered	Recovered
counts per min.	*counts per min.*	*per cent*
150	127	85
150	120	80
150	117	78
150	116	77
150	119	79
150	110	73
100	82	82
100	86	86
Mean		80
Maximum deviation from mean		7.0
Mean deviation from mean		3.8

* The plutonium was added to the sample after the ashing procedure.

TABLE III

Feces Blank Determinations by Calcium Oxalate Procedure*

Counts per min.†	Deviation from mean	Counts per min.†	Deviation from mean
0.3	0.63	1.9	0.97
1.7	0.77	1.1	0.17
0.6	0.33	0.9	0.03
0.9	0.03	0.7	0.23
1.6	0.67	0.6	0.33
1.9	0.43	1.2	0.27
0.5	0.43	0.9	0.03
0.6	0.33	0.7	0.23
0.5	0.43	0.9	0.03
0.0	0.93	0.2	0.73
1.7	0.77	0.5	0.43
1.7	0.77		
0.5	0.43		
0.9	0.03		
Mean		0.93	0.42

* Samples collected from persons having never been exposed to plutonium.

† Counts per minute. A counter background has been subtracted from each result. The counting time for each sample was 2 hours.

feces ash. The results are presented in Tables I and II. When known amounts of plutonium were added to a solution of the ash resulting from ashing a 50 gm. feces sample, the mean recovery was 80 per cent. The maximum deviation from the mean was 7.0 per cent and the mean deviation was 3.8 per cent.

TABLE IV

Recovery of Known Amounts of Plutonium from Samples of Human Feces by Calcium Oxalate-Cupferron Procedure

Pu added	Pu recovered*	Recovery
counts per min.	*counts per min.*	*per cent*
48.2	38.5	79.9
48.2	40.0	83.0
48.2	41.0	85.1
48.2	27.1	56.2
48.2	31.0	64.3
48.2	23.3	48.3
48.2	36.9	76.6
48.2	41.7	86.6
48.2	37.9	78.6
48.2	36.0	74.7
48.2	25.1	52.1
48.2	42.5	88.2
48.2	42.3	87.8
48.2	48.1	99.8
48.2	43.3	89.8
48.2	46.4	96.3
48.2	42.4	88.0
48.2	45.3	94.0
48.2	48.1	99.8
48.2	37.5	77.8
48.2	41.7	86.6
96.8	83.9	86.7
96.8	64.4	66.6
96.8	83.0	85.7
96.8	68.2	70.5
96.8	82.2	84.9
96.8	77.1	79.6
194	157.1	81.0
Mean		80.3
" deviation from mean		9.9
Maximum deviation from mean		32.0

* A counter background and a blank value of 0.93 count per minute have been subtracted from each result.

Feces samples were collected from persons having never been exposed to plutonium. Analysis of these blank samples by the calcium oxalate-cupferron procedure gave a small positive value even after correction for the background of the α-counter. The data are given in Table III. The mean blank value was 0.93 count per minute, and mean deviation from the mean 0.42 count per minute. This positive value may result from one or all of three factors: (1) α-activity in the feces as a result of the presence of naturally occurring α-emitters in the body and in the food consumed, (2) presence of naturally occurring α-emitters in the reagents, and (3) contamination of the sample during analysis.

The results given in Table IV were obtained when known amounts of plutonium were added to aliquots of human feces samples before ashing. They, therefore, represent the over-all recovery. These data show a mean recovery of 80.3 per cent. The maximum deviation from the mean was 32.0 per cent and the average deviation was 9.9 per cent.

SUMMARY

A detailed description is given of a method for determining plutonium in human feces. The essential features of the method are as follows: The dried sample is ashed in a muffle furnace and the ash dissolved in 4 M HCl. The reduced plutonium is carried from an aliquot of the feces ash solution with calcium oxalate. The calcium oxalate precipitate is digested with fuming HNO_3 and taken up in 4 M HCl. Ferric iron is added and the plutonium is extracted into $CHCl_3$ as the cupferride. The cupferride complex is destroyed with $HClO_4$. The plutonium is carried with LaF_3, transferred to a platinum plate, and counted in an α-counter. Results with solutions of both artificial and actual human feces ash show the recovery of plutonium by this method to be 80 per cent or better, with a mean deviation from the mean of 9.9 per cent.

ON THE NATURE OF THE PHOSPHORUS-CONTAINING LIPIDES OF CABBAGE LEAVES AND THEIR RELATION TO A PHOSPHOLIPIDE-SPLITTING ENZYME CONTAINED IN THESE LEAVES*

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In 1927 Chibnall and Channon (1) isolated a phosphatidic acid from cabbage leaves. Although in the present investigation a similar compound was obtained from *fresh* cabbage leaves, it was found that when the cabbage leaves were first subjected to steam treatment the phosphorus-containing lipide isolated was not a phosphatidic acid but one high in nitrogen content. These observations suggested to us that cabbage leaves, like the carrot (2, 3), contain an enzyme capable of splitting the phospholipide molecule at its ester linkage between the nitrogenous base and the phosphoric acid grouping. Such an enzyme has been identified in cabbage leaves, and some of its characteristics are described here.

Isolation of Phosphorus-Containing Lipides

From Fresh Cabbage Leaves—The thick mid-ribs of cabbage leaves were separated and discarded. The remainder of the leaves was then finely ground and 1000 gm. of the ground material transferred to a round bottom flask. A sufficient amount of a 3:1 alcohol-ether solution was added to provide 3 cc. of solvent per gm. of cabbage. The mixture was then refluxed for 12 to 14 hours at 55–60°. The solvent was decanted and filtered and the cabbage residue subjected to a second 14 hour extraction with the 3:1 alcohol-ether solvent. By means of reduced pressure (40 to 60 mm. of Hg) and in the presence of an atmosphere of CO_2 the combined extracts were concentrated at 55–60° to a volume of about 75 cc. The concentrated material was then reextracted several times with distilled petroleum ether (b.p. 30–60°) and the combined petroleum ether extracts brought to a volume of about 10 cc., this being carried out under reduced pressure and in an atmosphere of CO_2. The petroleum ether extract was carefully washed twice with 0.1 N HCl and then dried over Na_2SO_4. To the dried extract so obtained 2 to 3 volumes of acetone were added, and this was followed by the addition of several drops of a saturated solution of $MgCl_2$ in absolute alcohol. The precipitate that formed was washed repeatedly with acetone and then dissolved in a small volume of moist freshly distilled ethyl ether. The precipitation with acetone and $MgCl_2$ and the washing of the precipitate with acetone were repeated. The cabbage phospholipide obtained was dissolved in moist ethyl ether.

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* The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The fresh cabbage used here had a phosphorus content of 0.20 per cent of its dry weight, over 98 per cent of which was soluble in a 3:1 alcohol-ether mixture. Hydrolysis with 10 per cent alkali of the cabbage residue obtained after two alcohol-ether extractions yielded a negligible amount of ether-extractable material. The yield of phospholipide from fresh cabbage leaves was approximately 0.3 per cent.

From Steam-Treated Cabbage Leaves—Cabbage leaves from which the mid-ribs had been removed were placed on a wire tray and exposed to steam for 10 minutes. The material was then cooled, finely ground, and transferred to a round bottom flask. The material was then extracted exactly as described above. The yield of phospholipide from steam-treated cabbage leaves was approximately 0.3 per cent.

The cabbage residue obtained after two alcohol-ether extractions contained less than 2 per cent of the phosphorus present in the fresh cabbage leaves. Alkaline hydrolysis of this residue yielded only a negligible amount of "lipide" material.

From Dehydrated Leaves—2000 gm. of fresh cabbage leaves from which the mid-ribs had been removed were divided into two portions. One was steam-treated for 10 minutes, whereas the other portion was not exposed to steam. Both portions were then dehydrated at 60° for 10 hours. The final material contained approximately 10 per cent water. (Fresh leaves contain about 90 per cent water.) The dehydrated material was mixed with enough distilled water to raise its water content to 20 per cent; it was then finely ground and its phospholipides extracted as described above.

The following methods were employed for the analyses of the isolated phospholipides. Phosphorus was determined by King's method (4), choline by Glick's method (5), and total nitrogen by the micro-Kjeldahl method. The total glycerophosphoric acid content was measured by Burmaster's technique (6). The fatty acid content was obtained by alkaline hydrolysis of the phospholipide with 0.5 N alcoholic alkali, extraction of the acidified hydrolysate with petroleum ether, and subsequent evaporation and weighing of the petroleum ether extract. As a further check, the petroleum ether residue was dissolved in boiling alcohol and its fatty acid content determined with 0.020 N alkali. Free fatty acids were measured by the Fairbairn technique (7).

Some Characteristics of Phosphorus-Containing Lipides of Cabbage Leaves

Approximately 90 per cent of the petroleum ether-soluble phosphorus obtained from leaves was precipitated by acetone and $MgCl_2$. The phosphorus content of the phospholipide so obtained was not altered by repeated precipitations from its ether solution. Moreover, the amount of nitrogen present in the acetone-soluble fractions was very low. The isolated phospholipide was an amorphous, pasty substance which was soluble in petroleum ether, moist ethyl ether, and chloroform. It was insoluble in acetone and only slightly soluble in absolute or 95 per cent alcohol. The phospholipides obtained from steam-treated cabbage leaves were readily emulsifiable by water; those prepared from untreated cabbage leaves were not.

TABLE I

Composition of Phospholipides Obtained from Cabbage Leaves

Constituent	Batch I		Batch II	
	No treatment	Steam-treated	Dehydrated at 60°	Steam-treated, then dehydrated at 60°
Fatty acids, %	71	57	65	55
P, %	2.65	2.25	2.55	2.70
N, %	0.68	1.42	1.26	1.65
N:P, *molal ratio*	0.57	1.38	1.06	1.30
Choline, %	0	3.90	1.30	4.40
Choline:P, *molal ratio*	0	0.44	0.13	0.41
Glycerophosphoric acid, %	14.6	12.4	14.7	14.4

The composition of the various phospholipides isolated from cabbage leaves is shown in Table I. The phospholipides isolated from raw and steam-treated cabbage leaves differed in their nitrogen content. The ones obtained from the raw material had a lower nitrogen content than those from the steam-treated material. In keeping with our earlier observations on the carrot, we assumed that the phospholipide with a higher nitrogen content obtained from *steam-treated* cabbage leaves was the native phospholipide and that the one with the lower nitrogen isolated from *fresh* leaves resulted from enzymic action that occurred during the grinding and extraction of the cabbage leaves. The existence of an enzyme capable of splitting nitrogen from the phospholipide molecule was therefore sought.

Differences were also observed between the fresh and steam-treated cabbage leaves and between the dehydrated and steam-treated dehydrated cabbage leaves in the fatty acid contents of their phospholipides. No explanation is offered for these differences.

Phospholipide-Splitting Enzyme in Fresh Cabbage Leaves

Preparation of Crude Enzyme Extract—100 gm. of fresh cabbage leaves were ground and homogenized with 75 cc. of distilled water in a Waring blendor and the mixture allowed to stand at 5° for 1 to 2 hours. It was filtered through a Büchner funnel; the entire filtrate was then centrifuged. The supernatants or enzyme extracts used in the experiments described below were never older than 12 hours.

Measurement of Enzymic Activity of Crude Extracts—An ether solution containing 100 mg. of soy bean phospholipide was transferred to a 50 cc. glass-stoppered volumetric flask and the solvent removed by evaporation at 55–60°. The phospholipide was then mixed with 2.5 cc. of enzyme extract and 2.5 cc. of a phosphate buffer at pH 5.6, and the mixture vigorously shaken. 1 drop of chloroform was then added to the emulsified mixture as a preservative. The mixture was incubated at 25° for 2 hours. At the end of the incubation period, ethyl ether was added to the reaction mixture and the contents diluted to volume with ethyl ether and vigorously shaken. About 45 minutes were allowed for complete extraction. The ether-soluble and aqueous phases were then analyzed for nitrogen, choline, and phosphorus. The loss of ether-soluble nitrogen is the index of enzymic activity.

Table II

Specificity of Action of Crude Cabbage Extracts on Soy Bean Phospholipide

Substrate, 400 mg. of soy bean phospholipide in 0.05 M PO_4 buffer at pH 5.6. This phospholipide preparation contained 250 mg. of fatty acid, 12.4 mg. of P, 5.10 mg. of N, and 16.0 mg. of choline.

Constituent	Ether-soluble		Per cent change
	Original	After incubation for 21 hrs. at 25°	
P, *mg.*	12.4	11.8	5
N, *mg.*	5.10	3.33	35
Choline, *mg.*	16.0	8.4	47
Glycerophosphate P, *mg.*	11.8	11.7	0
Bound fatty acids,* *mg*	250	240	4
Choline:P, *molal ratio*	0.32	0.18	
N:P, *molal ratio*	0.90	0.62	

* Calculated as "oleic" acid.

Specificity of Action—The specificity of action of the cabbage enzyme is shown in Table II.[1] It is apparent that choline is liberated, but the choline split off the phospholipide molecule does not account for all of the nitrogen lost. The choline liberated from the phospholipide molecules during the incubation was found in a free form in the aqueous phase. This indicates that no degradation of the choline *per se* had occurred during cleavage of the phospholipide molecules. All of the nitrogen lost from the phospholipides was also found in the aqueous phase.

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[1] The period of incubation shown in Table II is 21 hours. For the determination of the pH curve and thermostability of the enzyme as described below the period of incubation adopted was 2 hours.

Table II shows that no glycerophosphoric acid was separated from the phospholipide molecule. Moreover, no increase in free fatty acids was noted. The loss of the nitrogenous base is apparently the only degradation suffered by the phospholipide molecule through action of this enzyme.

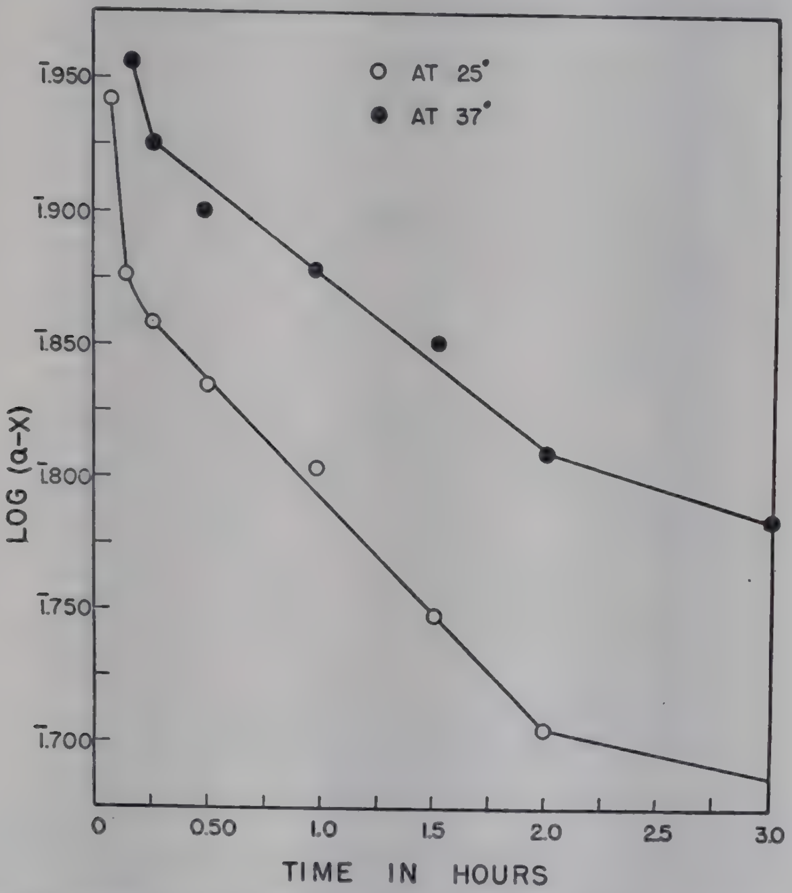

FIG. 1. Time-activity curves for phospholipide-enzyme mixtures in a 0.05 M phosphate buffer.

Kinetic Studies. Order of Reaction—Time-activity studies for this enzyme system, both in buffered and in non-buffered solution, yielded curves representative of a monomolecular reaction. This is shown in Fig. 1, where log $(a-x)$ is plotted against t, and where a represents the initial concentration of nitrogen in the intact phospholipide molecule, x represents the loss of ether-soluble nitrogen from the phospholipide, and t represents time.

The values for the reaction constant k, calculated from the equation $k = 2.3/t \log a/(a-x)$, were found to be constant between 0.5 and 2 hours at 25° ($k \times 10^3 = 2.0$); this indicates a first order reaction between these two intervals. It is evident that the reaction proceeds much faster during the first 0.5 hour than during later intervals, when, as already noted, it follows a first order reaction. Although a nearly straight line plot of log $(a-x)$ against time was obtained for incubation periods (at 25°) below 0.5 hour, the values for k were not constant. This was unexpected, but it might be explained by a preferential splitting of one of the nitrogenous bases. This reaction is quite fast, as evidenced by the finding that the amount of ether-soluble nitrogen cleaved in 2 hours was almost maximum (Fig. 1). These results are surprising when compared to those observed in a similar enzyme system identified recently in fresh carrots (3), which required 21 hours for maximum cleavage.

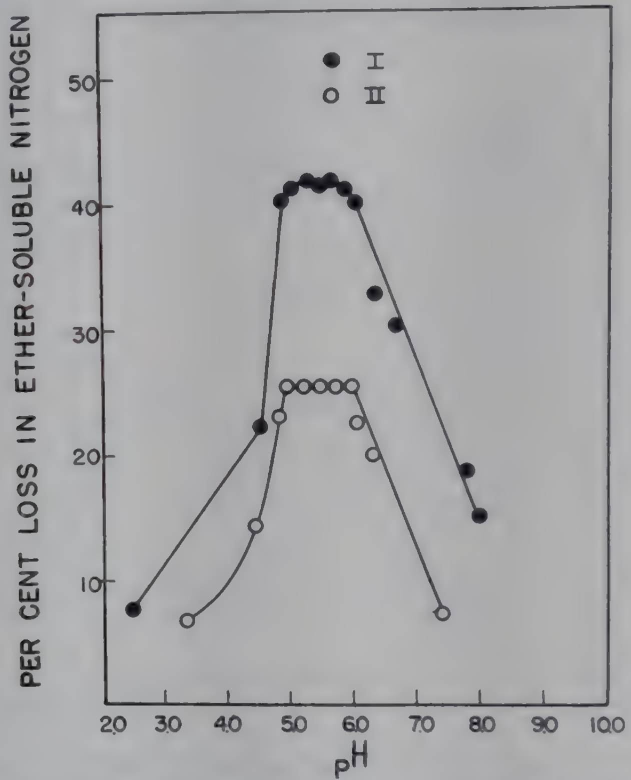

FIG. 2. Enzyme activity at various pH levels. The results of two experiments are shown.

The cabbage enzyme system was more active at 25° than at 37° in a 0.05 M phosphate-buffered solution at pH 5.6. The same relation of temperature to activity was reported for the similar enzyme isolated for the carrot. This could be due to the fact that the phospholipide emulsion is more easily broken at 37° than at 25°.

In view of the above considerations a 2 hour incubation at 25° was adopted as the standard procedure for the pH and thermostability studies described below.

pH Activity Curve—The procedure outlined above was followed. Mixtures of varying proportions of 0.10 M K_2HPO_4 and 0.10 M KH_2PO_4 and varying concentrations of H_3PO_4 were used to prepare solutions with the desired pH. The phospholipide-enzyme buffer emulsions were incubated at 25° for 2 hours. A plot of the activity of this enzyme system at various pH values is shown in Fig. 2. Optimum activity was observed at a pH range between 5.1 and 5.9. Little activity was found below pH 2.0 or above 8.0. Decreasing the incubation to less than 2 hours did not alter the pH range for the plateau, although the amount of activity was, of course, less than at 2 hours.

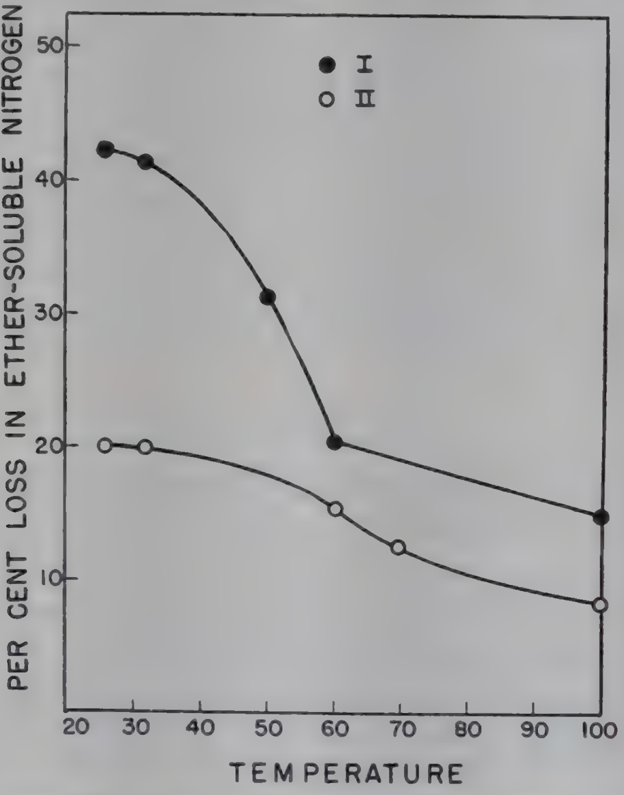

FIG. 3. Thermostability curve. The results of two experiments are shown

A pH curve was also obtained with a 0.05 M acetate buffer. Its characteristics were identical with those observed in the phosphate buffer.

Thermostability—10 cc. aliquots of the crude enzyme extract were heated at constant volume for 15 minutes at temperatures ranging from 25–100°. At the end of this time the extracts were immediately cooled to room temperature and the activity measured by the procedure outlined above. The results of this experiment are shown in Fig. 3, where activity is plotted against temperature.

This enzyme system is quite thermostable; it retained 30 to 40 per cent of its activity despite having been subjected to a temperature of 100° for 15 minutes.

DISCUSSION

The presence of an enzyme capable of attacking the linkage between the nitrogenous base and the phosphoric acid grouping of the phospholipide molecule accounts for the observed difference between fresh and steam-treated cabbage leaves in the nitrogen contents of their phospholipides. The activity of such an enzyme might explain the isolation of a phosphatidic acid from fresh cabbage leaves by Chibnall and Channon (1). The treatment to which they subjected the fresh cabbage leaves before extraction of the phosphorus-containing lipides, namely repeated grinding and subsequent heating to 70°, is conducive to enzymic action. The heat stability of this enzyme lends further support to this conclusion. Thus the dehydration of the raw cabbage leaves at 60° (without previous steam treatment) for 10 hours resulted in only partial inactivation of the enzyme (Table I).

SUMMARY

1. The characteristics and composition of the phosphorus-containing lipides from raw and steam-treated cabbage leaves are described.

2. The phosphorus-containing lipides isolated from raw and steam-treated cabbage leaves differed only in their nitrogen content; those isolated from raw cabbage leaves were characterized by a low nitrogen content and by an absence or low content of choline, while those isolated from steam-treated cabbage leaves were high in their nitrogen and choline content.

3. A phospholipide-splitting enzyme capable of attacking only the nitrogenous base-phosphoric acid linkage is shown to be present in fresh cabbage leaves. This enzyme had its maximum activity in a pH range from 5.1 to 5.9 in a 0.05 M phosphate buffer. It followed a first order reaction at 25° between 0.5 and 2.0 hours. The enzyme was quite thermostable, retaining 30 to 40 per cent of its activity after being exposed to a temperature of 100° for 15 minutes.

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CONCURRENT USE OF RADIOISOTOPES OF CALCIUM AND PHOSPHORUS IN THE STUDY OF THE METABOLISM OF CALCIFIED TISSUES*

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Important results have been obtained with tracer isotopes, particularly radiophosphorus, in the study of calcified tissues. Tracer studies with calcium have, however, been limited by the scarcity of the only suitable radioisotope of calcium, namely Ca^{45} with a half life of 180 days. The radiocalcium employed in previous studies (1–4) and in the present investigation was prepared with difficulty by bombardment of calcium metal with deuterons in a cyclotron (1, 2). At the present time it appears that insufficient amounts of radiocalcium will be available from neutron-induced nuclear transformations in the uranium pile for use in extensive studies with intact animals (5). Because of these circumstances it is unlikely that experiments such as that described in this paper can be extended in the near future. For this reason the work is now reported in its present state of progress.

Campbell and Greenberg (1) studied the distribution of Ca^{45} in the tissues and excreta of a rat following the intragastric administration of the isotope as the lactate. Their findings of a nearly equal specific retention of Ca^{45} by bones and teeth (pooled incisors and molars) on a dry weight basis does not give the correct impression as to the relative rates of turnover of calcium by bone and dental hard tissues. No distinction was made between the continuously growing incisor teeth and the non-growing molars nor between the enamel and dentin. Also, the Ca^{45} retentions were not recorded in relation to the calcium contents of the tissues. Incidental to another study Greenberg (4) examined the molar and incisor teeth of rats but did not separate the enamel and dentin of either type of teeth and again reported the results on a dry weight basis. The uptake of Ca^{45} by the femurs was greater than that of the molar teeth but was less than that of the incisors. On *a priori* grounds it would be expected that dental hard tissues would exchange calcium to a lesser degree than bone, a situation which has been demonstrated with respect to the exchange of phosphorus labeled with P^{32} (6, 7).

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The experiments reported in this paper were carried out in order to obtain data which would allow a comparison of the turnover of calcium in the normal calcified tissues and to compare in the same subject the turnover of calcium with that of phosphorus. To this end both Ca^{45} and P^{32} were administered to the animal and a chemical separation of calcium and phosphorus effected before the radioactivity measurements were made.

EXPERIMENTAL

A solution containing Ca^{45} and P^{32} was prepared by adding sufficient hydrochloric acid to a suspension of calcium lactate and sodium phosphate to dissolve the solids. 8 cc. of the solution containing about 0.6×10^6 counts per minute of Ca^{45} (122.3 mg. of Ca)[1] and nearly 9×10^6 counts per minute of P^{32} (6.7 mg. of P) were administered to a male rat weighing 238 gm. The syringe and stomach tube were washed and the Ca^{45} and P^{32} contents of the washings taken into account in the calculation of the actual administered doses of the radioisotopes. The animal was fed a normal diet and the urine and feces separately collected for 5 days, at the end of which period the animal was sacrificed and a blood sample obtained.

The contents of the intestine were added to the feces. The animal was skinned and the femurs and teeth removed. The remainder of the carcass (hereafter referred to as carcass residue) was ground in a food chopper and, with added water, converted into a suspension in a Waring blendor. Both epiphyseal ends of the femurs were cut off and the marrow removed from the diaphyses. The marrow, contained in a platinum dish, was extracted repeatedly with a mixture of alcohol and ether in equal parts. The epiphyses and diaphyses of the femurs were broken into fragments and extracted overnight in a Soxhlet apparatus with an alcohol-ether mixture. The molar teeth were pooled as one sample and the incisor teeth as another sample. After the teeth had been broken into bits and fat extracted, the enamel and dentin were separated by the method of Manly and Hodge (8). The serum proteins, after precipitation with trichloroacetic acid, were combined with miscellaneous fractions such as the red blood cells, the residue from the evaporation of the solvents of the alcohol-ether extractions, the fractions of the teeth not pure enamel or dentin, etc. This mixture was treated as a separate sample labeled débris. All samples, other than the protein-free serum, were dried and ashed at 500° to constant weight. The ash of each sample was dissolved in hydrochloric acid and diluted to a convenient volume. From this point all samples were treated in a uniform manner.

An excess of ammonium oxalate was added to an aliquot of the ash solution in a centrifuge tube and the resulting precipitate centrifuged. Ammonium hydroxide was added to the supernatant liquid until the pH was about 4 (brom-cresol green) and the solution of the newly formed precipitate centrifuged. The pH of the supernatant liquid was then carefully adjusted to 5 and the tube allowed to stand overnight and again centrifuged. The supernatant liquid and two washings of the precipitate were transferred to a volumetric flask. The contents of this flask, after being diluted to volume, were used for total phosphorus analyses and for P^{32} measurements.

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[1] The large dose of calcium given the animal was necessitated by the low specific ctivity of the preparation of Ca^{45}.

The precipitate of calcium oxalate contained in the centrifuge tube was dissolved in hydrochloric acid and reprecipitated by the addition of ammonium oxalate at pH 5. The supernatant liquid was poured off,[2] the precipitate again dissolved, and the calcium precipitated a third time as the oxalate. This precipitate was dissolved in hydrochloric acid and made to a convenient volume for subsequent determinations of total and radiocalcium.[3]

Phosphorus was chemically determined by the method of Fiske and Subbarow (9) adapted to the Evelyn photoelectric colorimeter. Radioactive phosphorus was measured with a dipping Geiger-Müller counter tube (10) coupled to a variable scaling unit. Calcium was determined chemically by the method of Clark and Collip (11). Radioactive calcium was counted in precipitates of calcium oxalate with a modified Libby (12) screen-walled counter. This counter tube was arranged so that a standard and a sample prepared in the same manner could alternately be brought under the sensitive region of the counter. In this way the activities of the standard and unknown were measured under identical conditions of counter characteristics. The background was determined, without opening the counter, by moving both samples away from the sensitive region of the counter.

Aliquots of the calcium solutions, after the determination of total calcium, were taken so as to contain 8 mg. of calcium.[4] Calcium oxalate was precipitated and collected by gentle suction on filter paper over a uniform area and washed with water and with acetone and ether. After the ether had evaporated, the precipitate and paper were transferred to the counting tube which was exhausted of air and left overnight in communication with a container of phosphorus pentoxide.

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[2] The supernatant liquids from the second precipitation of calcium oxalate from all samples except serum were examined for P^{32}. These liquids contained, on an average, 0.2 per cent of the P^{32} present in the supernatant fluid and washings of the first precipitation of calcium oxalate. It thus appears that essentially all of the phosphorus in the samples was present in the solutions employed for the analyses for total phosphorus and P^{32}.

[3] In an experiment in which a large amount of the P^{32} was added to a solution of nonradioactive calcium it was found that the calcium oxalate after three precipitations contained 0.0001 per cent of the original P^{32}. It thus appears that the treatment described in the text effectively separated the phosphorus from the calcium and assured that the observed activity of the calcium oxalate precipitates was not due to the contamination with P^{32}. This probability is further increased by the fact that the calcium was precipitated a fourth time (see the text) as the oxalate before the Ca^{45} was counted in the screen wall counter.

All radioactivity measurements were corrected for the resolving time losses and were calculated to a given date. An effort was made to keep the statistical error of the radioactivity measurements to less than 2 per cent of the observed counting rate but this was not feasible in the case of the measurements of Ca^{45} in serum, femur marrow, molar dentin, and molar enamel because of the low activities observed. The reproducibility of the technique of Ca^{45} determination is indicated by duplicate determinations of the activity of the calcium in feces, which agreed within 2.8 per cent. Duplicate Ca^{45} determinations in the urine agreed within 2.2 per cent.

RESULTS AND DISCUSSION

Of the administered radioisotopes 91.2 per cent of the Ca^{45} and 95.1 per cent of the P^{32} were recovered in the tissues and excreta of the animal. The lower recovery of Ca^{45} is possibly to be accounted for by the losses in the multiple precipitations of calcium oxalate. The feces contained 64.5 per cent of the administered Ca^{45} and 33.2 per cent of the P^{32}. The urine contained 2.05 per cent of the administered Ca^{45} and 7.42 per cent of the P^{32}. The large fecal output of both of the radioisotopes is probably a result of the very large amount of calcium which had to be administered. Campbell and Greenberg (1) who were able to give much less calcium as the lactate, with a higher specific activity than that employed in this study, found only 10.8 per cent of the isotope in the feces of a rat.

The results obtained with the tissues and fractions of the body are shown in Table I. Because of the unequal excretion of the isotopes and because of their unequal recoveries these data are not referred to the administered doses of the isotopes but are recorded as the actual recovered activities found in the several fractions of the animal and in the body as a whole. In the columns headed "Relative specific activity" the specific activities (*i.e.* counts per minute per mg. of P^{31} or Ca^{40}) of the various fractions are compared with the average specific activities of these elements in the body.

The results presented show that the uptake and retention of both P^{32} and

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[4] In certain cases such as serum calcium, bone marrow calcium, and standards it was necessary to add normal calcium to the solution to make the total 8 mg. before precipitating the calcium oxalate for counting.

Ca^{45} by calcified tissues stand in the following decreasing order:[5] bone marrow, skeletal epiphysis, skeletal diaphysis, incisor dentin, incisor enamel, molar dentin, and molar enamel. The higher uptake of the radioisotopes by the fractions of incisor teeth than by those of molar teeth is due in large part to the fact that the incisor teeth of the rat in contrast to the molar teeth are organs of continuous growth and eruption. The tracer isotopes present in the molar teeth were acquired by exchange of normal isotopes of the elements for the labeled isotopes. The incisor enamel and dentin acquired the tracer elements by the same process of exchange and also by deposition of newly formed mineral phase from a medium containing the radioelements.

TABLE I

Radiophosphorus and Radiocalcium Contents of Fractions of Rat Carcass

Fraction	Phosphorus			Calcium			Ratio of uptake and retention of P^{32} to Ca^{45}†
	Total P in fraction	Specific activity of P^{32}*	Relative specific activity	Total Ca in fraction	Specific activity of Ca^{45}*	Relative specific activity	
	mg.		*per cent*	*mg.*		*per cent*	
Skin and hair.......	37.0	6245.3	168	5.9	100.1	163	1.03
Carcass residue.....	1090.8	3763.1	101	2030.3	63.5	103	0.98
Serum..............	0.05	3494.3	94	0.14	84.2	137	0.68
Femur marrow......	0.56	5325.0	143	0.60	55.7	91	1.57
" epiphyses....	16.3	2927.7	80	38.9	56.7	92	0.86
" diaphyses....	56.8	2785.2	75	150.9	50.6	82	0.91
Incisor dentin.......	18.4	1940.9	52	37.9	33.8	55	0.94
" enamel......	3.3	992.4	27	9.8	16.4	27	1.00
Molar dentin........	5.3	536.0	14	13.2	7.2	12	1.16
" enamel.......	4.3	100.2	2.7	11.0	0.75	1.2	2.25
Débris.............	12.9	2907.1	78	17.8	74.6	116	0.67
Total body.......	1245.7	3712.5	100	2316.4	61.4	100	1.00

* Counts per minute per mg. of P.

† Ratios of relative specific activities of P^{32} and Ca^{45}.

The figures given in the last column of Table I are not a measure of the absolute relationship of P^{32} and Ca^{45} uptake. Knowledge as to the number of phosphorus atoms turned over in relation to the number of calcium atoms turned over cannot be obtained from this experiment because there is no way of knowing the manner in which the specific activity of the plasma inorganic phosphate and ionized calcium varied with respect to one another. These calculated results do afford a comparison of the relative uptake of

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[5] Except for Ca^{45} uptake in marrow and epiphysis which was nearly identical.

P^{32} and Ca^{45} by the tissues studied in relation to the average distribution of the isotopes throughout the body. Since the apatite mineral phase is essentially the same in all calcified tissues (13, 14), the difference in rates of exchange of calcium and phosphorus is probably due to physiological or structural factors which produce differences in rates of migration of calcium and phosphate ions into the various calcified tissues. A suggestion that such factors operated is seen from the fact that the relative uptake of P^{32} to Ca^{45} by the calcified tissues stands in an inverse relationship to the order of the uptake of the radioelements.

SUMMARY

Methods have been presented for the separation of radiocalcium and radiophosphorus in tissues which allow the concurrent use of these isotopes. The uptake and retention of both radiocalcium and radiophosphorus by calcified tissues stand in the following decreasing order: femur marrow, femur epiphysis, femur diaphysis, incisor tooth dentin, incisor tooth enamel, molar tooth dentin, and molar tooth enamel. Evidence has been presented that under conditions *in vivo* calcified tissues do not all exchange calcium and phosphorus in the same proportion.

We wish to express our thanks to Dr. D. M. Greenberg who generously shared his supply of radiocalcium with us and to Dr. E. O. Lawrence who donated the radiophosphorus used in this study.

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THE COMBINATION OF ORGANIC ANIONS WITH SERUM ALBUMIN

IV. STABILIZATION AGAINST UREA DENATURATION

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Recent investigations have shown that sodium caprylate and related compounds have great influence upon thermal stability (1–3), electrophoretic mobility (4), rate of papain digestion (5), and urea denaturation (6–8) of bovine and human serum albumin. The combination of certain organic anions with native bovine albumin has recently been investigated by the equilibrium dialysis technique by Klotz *et al.* (9) and Teresi and Luck.[1]

The present investigation is an extension of the work of Boyer *et al.* (7) on urea denaturation. A number of compounds of related structure have been examined for ability to prevent the viscosity rise which occurs when a mixture of albumin and 6 M urea is allowed to stand at room temperature.

EXPERIMENTAL

Amorphous and crystalline bovine serum albumin were employed in most of the experiments. These albumin preparations were obtained from the Armour Laboratories through the courtesy of Dr. J. D. Porsche. Many of the compounds were previously used in the cloud point studies (3). Compounds were Eastman highest purity or the equivalent. The hexylmalonic acid was synthesized by Dr. Mark Neuhof according to the method of Dox (10). Its melting point and equivalent weight were 104° and 95.4 respectively. All compounds were used as their sodium salts.

The Ostwald type viscosimeters used were permanently clamped on a brass support which fitted in vertical position in an aquarium type water bath. Unusual precautions were observed to keep the viscosimeters clean and all solutions free of particulate matter. After use, the viscosimeters were immediately cleaned with Nacconol N. R. S. F. or Dreft solution, followed by thorough rinsing with dilute acetic acid and with water. The bank of viscosimeters was then inverted and a stream of dry air directed through them. An infra-red lamp increased the speed of drying. The viscosimeters used had water flow times of 60 to 90 seconds at 30°, with capillary lengths of 10 cm. and diameters of approximately 0.5 mm. With 5 ml. samples, the heads of pressure were 12 to 16 cm. of water.

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[1] Teresi, J. D., and Luck, J. M., unpublished.

The bovine albumin was prepared in a concentrated stock solution by long stirring with distilled water, followed by the slow addition of 1 N NaOH until all the albumin had dissolved and the pH of the 20 per cent solution was 7.5 to 7.7. Later it was found that the vacuum reconstitution of albumin was more convenient, yielding 20 per cent solutions in distilled water, which then were adjusted to the pH values indicated. This method consists of pumping the air from the container of the dry albumin and water with a vacuum pump, then mixing the powder and water just before release of the vacuum. If a few clumps of albumin remain, they may be broken up by application of a vacuum for an instant, although some foaming occurs at this time. The remaining albumin quickly dissolves if the solution is now stirred. Solutions of crystalline bovine albumin prepared in this way are clear even before addition of base. This method of stock solution preparation was used for the work with crystalline albumin and for the stock solutions used in the intrinsic viscosity determinations.

After pH adjustment, the stock solutions were filtered under pressure and diluted to 20.0 per cent by weight[2] on the basis of dry weight determinations. The drying was carried out at 105° for 24 hours. Fresh albumin solutions were prepared every 10 days and stored in the cold room.

Mixtures of 9 M urea, phosphate buffer of pH 7.5, sodium chloride, water, and the substance under investigation were prepared. After mixing, the desired volume of stock albumin was added to each solution. Final solutions were stirred magnetically and set aside for 24 hours at room temperature. 5 ml. samples of each solution were measured into dry viscosimeters in a water bath at 30.0°. 15 minutes later the flow times were measured to the nearest 0.1 second. Except in specific instances the sodium chloride concentration was kept at 0.01 M. The usual ionic strength ($\Gamma/2$) due to phosphate buffer and sodium chloride was 0.08.

RESULTS AND DISCUSSION

The results obtained with a series of aromatic carboxylates are given in Fig. 1. Since the experiments with amorphous albumin served to indicate the compounds of special interest for later studies with crystalline albumin, the curves are drawn through points uncorrected for the increased viscosity of the medium due to the indicated organic salts in the absence of protein. The curves would each be lowered about 1 per cent at 0.02 M and 7 per cent at 0.06 M if these corrections were applied.

Benzoate and acetyltryptophan raise the viscosity of the system above the control[3] at all concentrations above 0.02 M. The introduction of nitro or hydroxyl groups into the benzene ring leads to compounds with ability to keep the viscosity of the system below that of the control.[3] For comparison, the portion of the caprylate curve in this viscosity range is included. It should be mentioned that benzoate and the salicylates have been used in experiments with crystalline bovine albumin (see Fig. 4). In the latter experiments, benzoate is as active as phenyl acetate in preventing the rise of viscosity. No explanation is offered for this divergence, except that the crystalline albumin contains less contaminating globulin.

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[2] The concentration of albumin in the various experiments is given in per cent, which in this instance means gm. per 100 ml. of solution.

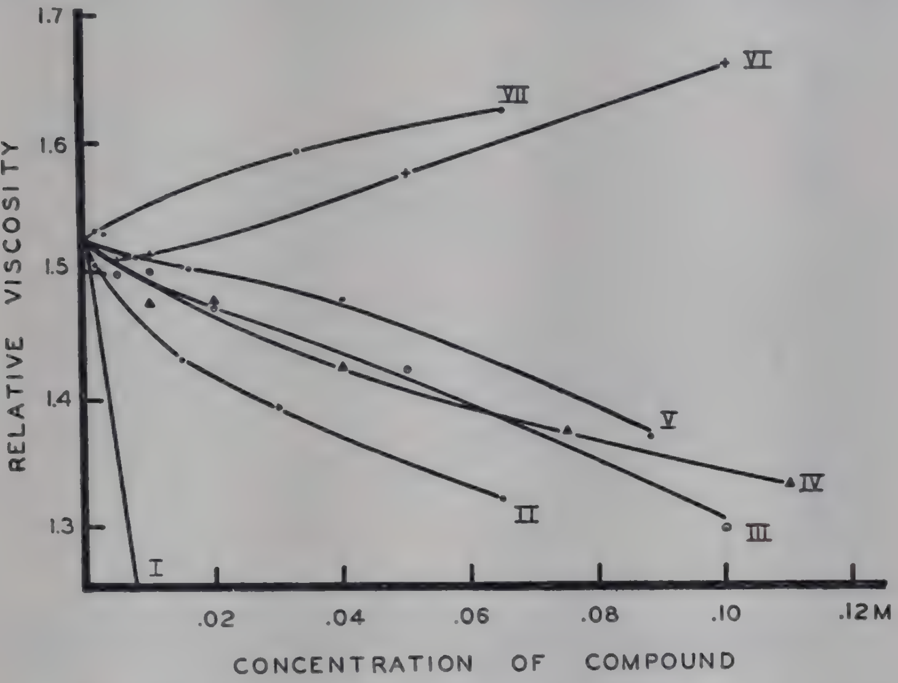

Fig. 1. The influence of various aromatic anions upon the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.01 M NaCl, and 2.1 per cent bovine serum albumin. The viscosity values are relative to 6 M urea containing salt and buffer. Curve I, sodium caprylate (given for comparison); Curve II, sodium acetyl salicylate; Curve III, sodium salicylate; Curve IV, sodium 3,5-dinitrobenzoate; Curve V, sodium *m*-nitrobenzoate; Curve VI, sodium benzoate; Curve VII, sodium acetyltryptophan.

The results for a series of sulfonates are included in Fig. 2. These data are also from experiments with amorphous albumin and the points have not been corrected for the viscosity rise with increasing concentration of organic salt in the organic salt-urea system. Sodium benzenesulfonate and disodium sulfosalicylate are seen to raise the viscosity of the system markedly. Valeryl sulfonate and sulfanilate have little or no effect. 4-Chlorobenzenesulfonate and 2,5-dichlorobenzenesulfonate have increasing ability to keep the viscosity of the system below that of the control.[3] Several of these substances have also been studied with crystalline albumin.

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[3] The control solution is the mixture of albumin, 0.01 M NaCl, 0.01 M phosphate buffer, and 6 M urea, but free of added organic salt. Such solutions yield relative viscosity values which are plotted upon the relative viscosity axis in the various figures.

Fig. 3 presents the viscosity data for acetate, chloride, and the chloroacetates. Each viscosity value is calculated with reference to the protein-free system as the standard. The chloroacetates are able to keep the viscosity of the systems containing crystalline bovine albumin below that of the control.[3] At concentrations above 0.05 M, trichloroacetate keeps the viscosity of the system below 1.20. The relative viscosity at 0.11 M trichloroacetate approaches 1.10, the value estimated to represent the system, native albumin-6 M urea. Monochloroacetate and dichloroacetate are seen to be less effective in keeping the viscosity at low values.

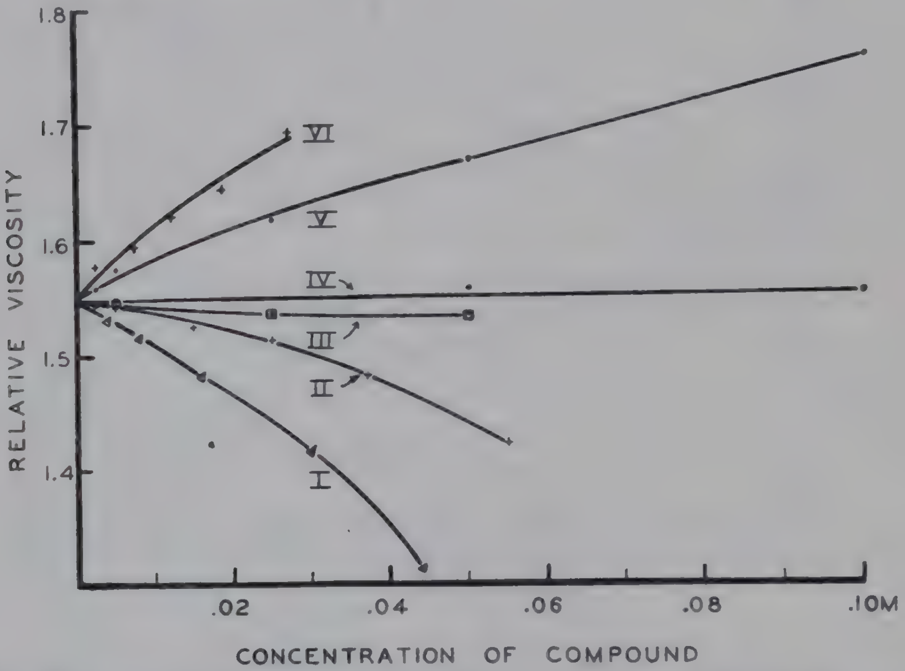

Fig. 2. The influence of various sulfonates upon the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.01 M NaCl, and 2.1 per cent bovine serum albumin. The viscosity values are relative to 6 M urea containing the salt and buffer. Curve I, sodium 2,5-dichlorobenzenesulfonate; Curve II, sodium *p*-chlorobenzenesulfonate; Curve III, sodium valeryl sulfonate; Curve IV, sodium sulfanilate; Curve V, disodium sulfosalicylate; Curve VI, sodium benzenesulfonate.

The corrected viscosity data for benzoate and various related compounds are presented in Fig. 4. The system is identical with that of the experiments recorded in Fig. 3. It is apparent that benzoate and phenyl acetate have much the same effect, while phenyl butyrate is as effective as caprylate. The modified benzoates, salicylate and acetyl salicylate, have greater effects than benzoate at any chosen concentration. The benzoate derivatives and phenyl acetate require concentrations of 0.08 M or greater to keep the viscosity of the system below 1.20. Cinnamate (phenyl acrylate) is seen to have stabilizing properties intermediate between those of phenyl acetate and phenyl butyrate. The compound, hexyl malonate, which possesses a structure related to caprylate, except that two carboxyl groups are present at one end of the molecule, is seen to be almost as effective as caprylate. This compound is an exception to the observation that divalent anions do not stabilize.

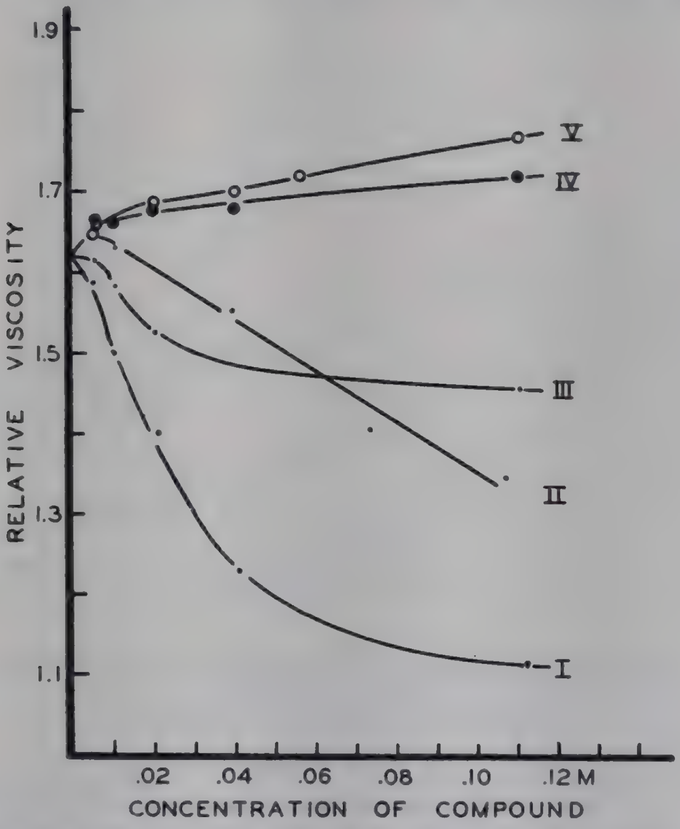

FIG. 3. The influence of anions of related structure upon the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.01 M NaCl, and 2.1 per cent crystalline albumin. The viscosity values are relative to solutions containing all components except protein. Curve I, sodium trichloroacetate; Curve II, sodium dichloroacetate; Curve III, sodium monochloroacetate; Curve IV, sodium chloride; Curve V, sodium acetate.

Fig. 5 presents the viscosity relationships for the interactions between albumin and sodium dodecyl sulfate (SDS) in the presence and absence of 6 M urea. For purposes of comparison all the corrected relative viscosities were recalculated with respect to distilled water as a standard. When necessary (6 M urea solutions), a constant density correction was applied.

Thus density changes due to the inclusion of the albumin or to the increasing SDS concentration were neglected.

The original experiments were carried out to determine the effect of a known denaturant upon the viscosity of the albumin-urea system. Fortunately concentrations of dodecyl sulfate were chosen which indicated the minimum in the bovine albumin curve (see Curves IVa and IVb, Fig. 5). Curves II and IVa are analogous in that both represent viscosity changes for amorphous bovine albumin. Curve II indicates the changes which occur with an increasing concentration of dodecyl sulfate when no urea is present. Curve IVa presents the data for the 6 M urea system. It is interesting that the viscosity of the aqueous system (Curve II) did not begin to increase at dodecyl sulfate concentrations at which the protection against urea denaturation is evident. The minimum for Curves IVa to IVc is evidenced at a dodecyl sulfate concentration corresponding to a mole ratio of 8 or 9 moles per mole of albumin. After that concentration is exceeded, the viscosity of the system rises steeply to points much higher than the control (no SDS). The curve for amorphous human albumin is

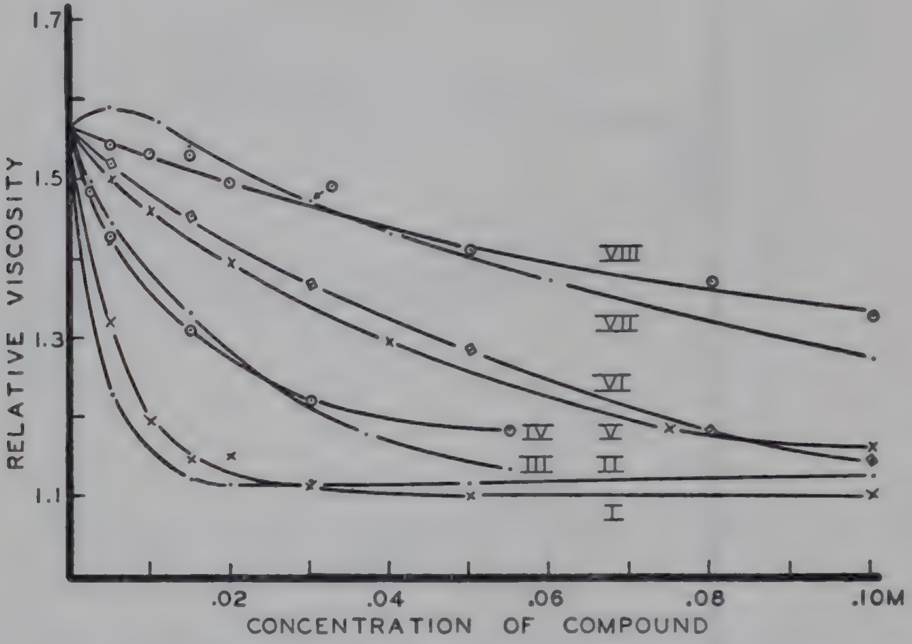

FIG. 4. The effect of various aromatic anions upon the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.01 M NaCl, and 2.05 per cent crystalline albumin. The viscosity values are relative to solutions containing all components except protein. Curve I, sodium phenyl butyrate; Curve II, sodium caprylate (for comparison); Curve III, disodium hexyl malonate; Curve IV, sodium cinnamate; Curve V, sodium acetyl salicylate; Curve VI, sodium salicylate; Curve VII, sodium phenyl acetate; Curve VIII, sodium benzoate.

remarkably similar to that of the bovine, except that the control begins at a lower relative viscosity (1.9 rather than 2.3). Thus each of the curves has a minimum viscosity at 0.0025 M SDS and a characteristic point where the viscosity is identical with that of the solution lacking SDS. The human albumin system reaches this point at 0.011 M SDS (mole ratio = 39:1), while the curve for crystalline bovine albumin reaches this point at 0.020 M SDS (mole ratio = 69:1).

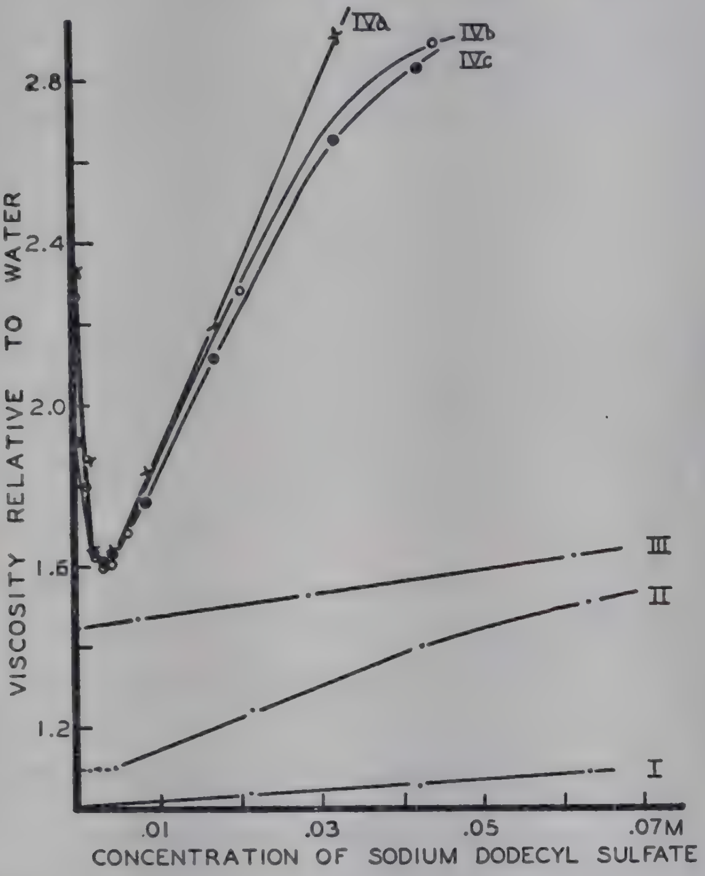

Fig. 5. The effect of sodium dodecyl sulfate (SDS) upon the urea denaturation of human and bovine serum albumin. Curve I, SDS in water; Curve II, SDS in 2.1 per cent bovine albumin solution (amorphous preparation); Curve III, SDS in 6 M urea solution; Curve IVa, SDS in 6 M urea containing 2.1 per cent amorphous bovine albumin; Curve IVb, SDS in 6 M urea containing 2.1 per cent crystalline bovine albumin; Curve IVc, SDS in 6 M urea containing 2.1 per cent amorphous human albumin. All relative viscosity values of Curve II were calculated with respect to water containing the same concentration of SDS. The viscosity values of Curves IVa, IVb, and IVc are corrected for the viscosity increment due to the SDS present. The viscosity values plotted for Curves III and IV were corrected for the increased density of the 6 M urea solution.

Neurath and Putnam (11, 12) found that equine albumin formed several discrete complexes with dodecyl sulfate at pH 6.7. The first complex, at a mole ratio of 55:1, exhibited an intrinsic viscosity identical with that of the native albumin. A second complex of increased intrinsic viscosity was evident when the mole ratio of detergent to albumin was increased to 110:1. These workers assumed, because of the lack of precipitability of the barium salt of dodecyl sulfate, that the detergent was completely bound until the mole ratio of 110:1 was exceeded. Similar complex formation between egg albumin and alkyl sulfonates or sulfates has been investigated by Lundgren and coworkers (13–15), using several experimental techniques. The data in Fig. 5 would seem to indicate that less than 9 moles of SDS are bound by each molecule of human or bovine albumin to form a complex which is stable toward the denaturing action of urea.

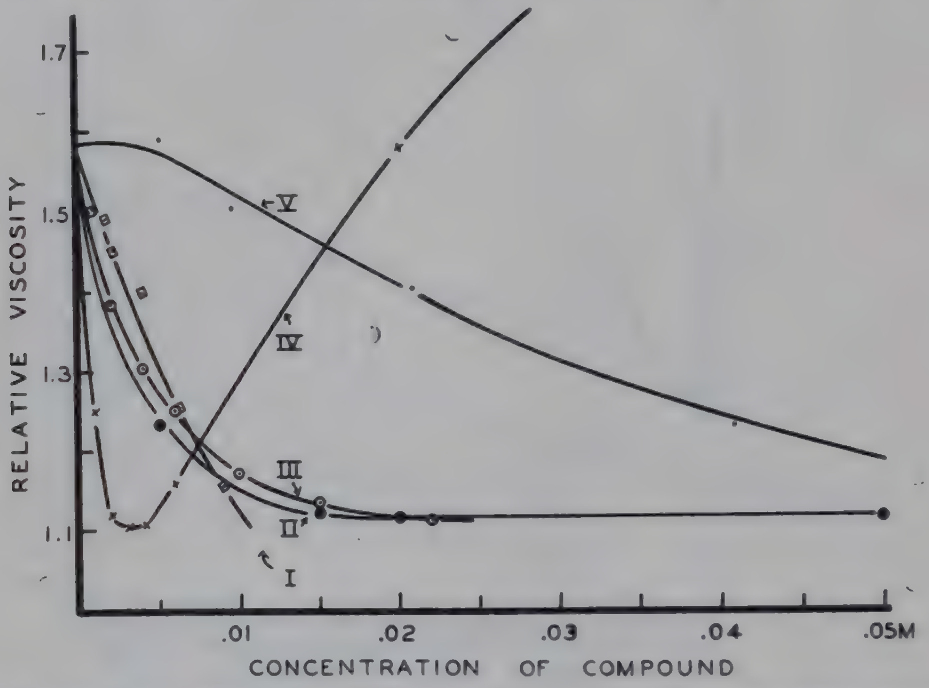

FIG. 6. The comparative effects of several "stabilizing" anions upon the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.01 M NaCl, and 2.1 per cent crystalline bovine albumin. The viscosity values are relative to the 6 M urea containing the salt and buffer. Curve I, sodium 2,4-dichlorophenolate; Curve II, sodium caprylate; Curve III, sodium picrate; Curve IV, sodium dodecyl sulfate; Curve V, sodium trichloroacetate.

Fig. 6 presents comparative data for the compounds most effective in preventing the viscosity rise in the albumin-urea system. The curves for sodium picrate and sodium dichlorophenolate have not appeared in the previous figures. It is well to consider picrate more fully, since comparative data were obtained in the amorphous bovine albumin-urea system for several related compounds. Picrate was found to be the most effective nitrophenolate, the other compounds giving curves similar to those of salicylate or benzoate. The compounds investigated were those having a nitro group in the 2, 3, or 4 position, or in the 2 and 4 positions. The 2,4-dinitrophenolate was the most effective of these compounds. Disodium flavianate, which has sulfonate and nitrophenolate groupings, was almost without effect on the viscosity of the system up to 0.02 M. The low solubility of these compounds makes it difficult to reach high ratios of compound to albumin, at least when the albumin is maintained at 2 per cent concentration. Another complicating factor is that of the variance in pK values for these compounds. The pK values for these nitrophenolates vary from 8.3 for the meta (3-) compound to less than 1 for picrate. Stabilization is not simply a function of pK (*i.e.*, of the actual anion concentration), since 2,6-dichlorophenolate (pK 6.8) is a stabilizer, while 2-nitrophenolate (pK 7.1) or 2,4-dinitrophenolate (pK 4.1) is not effective in equivalent concentrations. There is an increase in the apparent pH of the urea-albumin solutions as increasing amounts of such salts of weak acids are introduced, since the ionic concentration of dihydrogen phosphate is of the order of 0.005 M, but at 0.01 M concentration of dichlorophenolate, for example, the pH increase would not account for the prevention of viscosity rise.

The curves in Fig. 6 show similar behavior in approaching $\eta_R = 1.1$ as a limiting value, except the curve for dodecyl sulfate which reverses direction above 0.05 M concentration. The compounds may be compared according to the concentration needed to maintain the viscosity of the albumin-urea system below 1.15 or 1.20. Dodecyl sulfate is seen to require the lowest concentration ratio (moles per mole), followed by the other compounds as listed in Table I. Sodium laurate, with a chain length close to that of dodecyl sulfate, follows the phenyl butyrate curve up to 0.04 M concentration, after which its curve rises steeply. Above 0.04 M concentration, such solutions show increasing opalescence, and so the viscosity data above that concentration are uncertain.

A number of other compounds have been investigated for their ability to prevent the normal viscosity rise in urea-albumin solutions. Polymerized metaphosphate (Calgon), oxalate, adipate, phthalate, and sulfosalicylate increase the viscosity of the system above that of the control (urea-albumin solution without added organic salt). The rise in relative viscosity at a particular concentration of organic salt is greater than that expected from the viscosity increments shown by the organic salt in protein-free urea solutions. Butyrate and valeryl sulfonate change the viscosity of the system inappreciably. Phenoxyacetate and hippurate had little or no effect upon the viscosity of the system, contrary to what one might expect if it were assumed that a certain chain length following a carboxyl group was all that was required to prevent the viscosity rise of the albumin-urea solution.

The introduction of sodium chloride, sufficient to increase the concentration to 0.21 M rather than 0.01 M, leads to a higher relative viscosity in the control solution (1.84 compared to 1.59) but does not modify the location or shape of the curves for caprylate or trichloroacetate.

TABLE I

Comparison of Compounds for Ability to Prevent Viscosity Increases Due to Urea Denaturation

Compound	Mole ratio* of compound-albumin at			Cloud point temperature (mole ratio, 7:1)	
	$\eta_R = 1.20$	$\eta_R = 1.15$	$\eta_R = 1.12$		
				°C	
Sodium dodecyl sulfate	4	6	7	72.5†	75.7‡
" caprylate	23	34	51	70†	73‡
" 2,4-dichlorophenolate	27	32	35–60		
" picrate	27	41	60		
" phenyl butyrate	34	53	85	69†	
Disodium hexyl malonate	109	157		66§	
Sodium cinnamate	127				
" trichloroacetate	162	232	323	67†	71‡
" salicylate	224	680			
" acetyl salicylate	249	324			
" phenyl acetate	440			67†	
" dichloroacetate					69.2‡
" monochloroacetate					68‡
" acetate				64.5†	

* The value for molar concentration of albumin was calculated from the known concentration of crystalline bovine albumin and the molecular weight of 70,000. The molar concentration of a compound at the indicated η_R values was obtained by interpolation of the corrected viscosity-concentration curve.

† 25 per cent crystalline human albumin containing 0.15 M NaCl. The cloud point of the albumin-salt mixture (lacking organic salt) was 64°.

‡ 15 per cent crystalline bovine albumin containing 0.15 M NaCl. The cloud point of the albumin-salt mixture (lacking organic salt) was 67.5°.

§ 15 per cent human albumin containing 0.15 M NaCl. The cloud point of the albumin-NaCl mixture was 65°.

Intrinsic Viscosity Determinations—The slope of the curves of relative viscosity *versus* albumin concentration for low concentrations of protein is of more significance than the relative or absolute viscosities of a constant albumin, variable "stabilizer" concentration system. However, the curves given in the previous figures are necessary preliminaries to determine the compounds and concentrations for further investigation. The experimental work and treatment of the data obtained follow the treatment given by Neurath and Putnam (12). These workers determined curves for rela-

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tive viscosity *versus* total solute concentration for several mixtures of dodecyl sulfate and equine albumin in aqueous buffer solutions.

The estimation of intrinsic viscosity values required the determination of relative viscosities at several albumin concentrations, a constant mole ratio of organic salt to albumin being maintained in each series. The procedure consisted of mixing the stock albumin solution and sodium dodecyl sulfate solution in desired proportions. This mixture was then added to the urea-buffer-salt mixture to attain the desired concentrations of urea and sodium chloride, together with a known concentration of albumin. The mole ratio of SDS to albumin remained constant, although the actual concentration of both diminished together.

Fig. 7 presents several curves which yield intrinsic viscosity values as the intercept on the ordinate axis. The terminology and symbols are analogous to those used by Neurath and Putnam. The relative viscosity values, for the solutions containing 0.01 M sodium chloride, were calculated with respect to the urea-buffer-salt solvent, the volume occupied by the SDS and the viscosity increment due to the small amount of SDS present being ignored. The highest concentration of SDS in the solutions which provide the data for Fig. 7 was 0.004 M for the case of 1.6 per cent albumin with a mole ratio of SDS-albumin of 18:1. Corrections for the viscosity contribution of the "stabilizer" and the concentration employed would be necessary before calculation of the η_{sp} values (where $\eta_{sp} = \eta_R - 1$), for the cases in which high mole ratios of stabilizer to albumin are employed.

Fig. 7 indicates that the dodecyl sulfate maintains the $\eta_{sp}:C$ ratio at lower values than those observed in the absence of stabilizer at all concentrations of albumin used. The weight intrinsic viscosities obtained from extrapolation of the curves of Fig. 7 to the ordinate axis are 13.5, 4.8, and 8.2 for the albumin in the absence of stabilizer, for albumin containing SDS in a mole ratio of 18:1, and for albumin containing SDS in a mole ratio of 9:1, respectively.

The results previously presented, as well as those of Boyer *et al.* (7), indicate that stabilization against urea denaturation is a property of only a few of the many compounds examined. For the purposes of this discussion, stabilization is defined as follows: the ability of a compound to prevent the normal rise in viscosity which occurs when an albumin solution that contains urea is allowed to stand. Specifically, the stabilizers among the compounds examined by the present technique are those substances which maintain the relative viscosity of a 2 per cent albumin-6 M urea solution at 1.10 to 1.20 for a period of 24 hours. Thus, the definition limits this discussion to the first eight or ten compounds listed in Table I.

The comparative efficacy of stabilizers would then be determined by the mole ratio necessary to keep the viscosity of the albumin-urea system at its lowest value. Thus, dodecyl sulfate is the most effective stabilize listed. Trichloroacetate may be considered to be a stabilizer, but the othe chloroacetates are ineffective.

A chain length of 7 or more carbon atoms seems necessary in order t give a fatty acid salt the property of stabilization. The chain lengt necessary for alkyl sulfates has not been determined. Phenyl butyrat

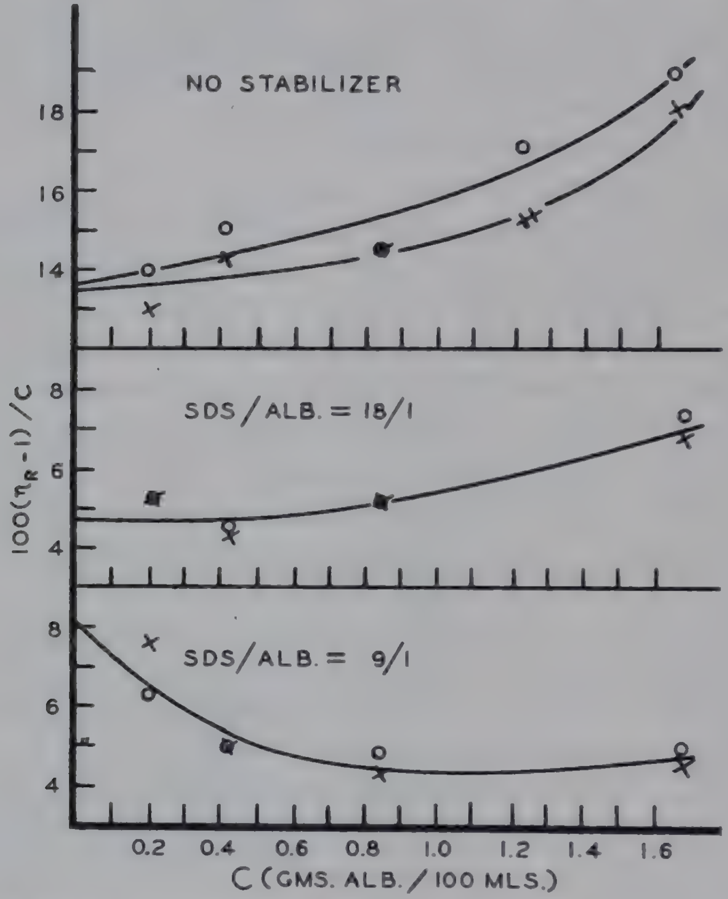

FIG. 7. The effect of constant ratios of dodecyl sulfate to albumin upon the slope of the relative viscosity *versus* albumin concentration. All solutions contained 6 M urea and 0.01 M phosphate buffer (pH 7.5). ○ corresponds to solutions containing 0.21 M NaCl; × data from solutions containing 0.01 M NaCl. The molar ratios of SDS-albumin are indicated on the graph.

and cinnamate are more effective than phenyl acetate, indicating that more than 1 carbon atom is necessary in the aliphatic portion of the anion. The reason for the ineffectiveness of phenoxyacetate or hippurate as stabilizers is not readily apparent, since they possess the chain length necessary. One tentative explanation is that their aliphatic chain portions are more polar in character, and so their ability to stabilize and to combine with albumin would be comparable to that of benzoate rather than to phenyl butyrate.

The ability of picrate, dichlorophenolate, and trichloroacetate to stabilize cannot be explained on the assumption that a non-polar chain is required in the molecule. Furthermore, the effect of the addition of polar but uncharged groups to compounds otherwise ineffective (compare salicylate or acetyl salicylate with benzoate, etc.) is yet to be explained.

The tentative hypothesis may be advanced that combination of these particular anions with positively charged groups on the albumin molecule produces a complex which is resistant to the action of the urea. The resistant complex responds to the urea treatment with little viscosity increase. Such a complex would show hydrolysis rates approaching those of native albumin when subjected to papain hydrolysis. Experiments on papain hydrolysis are of course complicated by the possibility of inhibition of papain action by the stabilizer in question.

It is difficult to conceive of stabilization occurring without the bound molecules forming a link, however weak, between neighboring peptide chains of the albumin molecule. Such links might involve, besides the electrostatic bond, a non-polar interaction with hydrocarbon residues of chains or with the residues of other bound stabilizer molecules. This hypothesis would explain the action of such compounds as caprylate or phenyl butyrate. For the phenolates and trichloroacetate, the second linkage may be a hydrogen bond. The contrasting behavior of monochloroacetate and trichloroacetate or the individual behavior of the various phenolates would require further explanation.

The viscosity data presented give no indication of the number of molecules of a particular compound which combine to produce an albumin complex resistant to urea denaturation. The amount of binding of several of these active anions by albumin is being investigated by the equilibrium dialysis technique. Differences in binding energy and in maximum combination number are apparent between such compounds as the mononitrophenolates and picrate.[1] The advantage of the viscosity work is that it gives an indication of the compounds which may be presumed to be active and of the mole ratios at which activity may be expected. The minima in the curves of Fig. 5 indicate that less than 8 molecules of dodecyl sulfate combine with albumin to produce the stable complex. Further work will be necessary to establish the actual number of molecules bound in the formation of the stable complex for each of the active stabilizers.

The ability of such compounds to prevent denaturation will depend upon the denaturant employed. Boyer *et al.* (7) found that caprylate would prevent increases of viscosity in 2.5 M guanidine hydrochloride, but would not prevent increases in 6 M guanidine hydrochloride. Similarly, we have found that 0.05 M caprylate will not prevent the viscosity increase which occurs when a solution containing 2 per cent albumin and 1.2 per cent SDS is allowed to stand.

Certain conclusions may be drawn concerning the comparative effectiveness of these compounds against denaturation by heat and by urea. Th determination of the thermal stability of albumin solutions containing a number of organic anions has demonstrated that the cloud point increase with increase in chain length when members of a homologous series o compounds were investigated (*cf.* (1–3)). However, it is apparent from the viscosity experiments that only a few compounds among those which raise the cloud point are effective in preventing urea denaturation. Butyrate is able to raise the cloud point of 25 per cent human albumin from 64° to 75° when introduced in 0.30 M concentration (mole ratio 81:1); butyrate is ineffective in preventing viscosity increases in 6 M urea up to a mole ratio of 337:1.

Compounds which stabilize albumin against urea denaturation presumably will greatly increase the cloud point of albumin solutions. Thus dodecyl sulfate, caprylate, phenyl butyrate, and trichloroacetate, each in 0.015 M concentration (mole ratio 7:1), raise the cloud point of 25 per cent human albumin solutions from 64° to 72.5°, 70°, 69°, and 67°, respectively. The cloud point data for many of the compounds listed in Table I are included in the last column.

Moderate concentrations of several of the organic salts result in the formation of gels at the temperatures to which the albumin is subjected during the measurement of the cloud point. Gels are also obtained when 25 per cent albumin solutions containing stabilizers are subjected to heating at 57° for several months. Gel formation would result from heat denaturation if an added salt interfered with aggregation of the denatured protein but did not stabilize the native protein.

True stabilization would involve the maintenance of albumin in the native state as measured by several independent criteria, such as unchanged solubility, unchanged intrinsic viscosity, unchanged digestion rate, and unchanged molecular weight. Considering the data on stabilization thus far available, sodium caprylate would seem to stabilize these albumins in the native form as measured by the second and third criteria mentioned. This conclusion would be dependent upon the demonstration of no inhibition of papain by caprylate. The evidence is not complete for phenyl butyrate, dodecyl sulfate (low mole ratios only), or trichloroacetate, but tentatively we assume them to be true stabilizers. Evidence is incomplete for the phenolic compounds, picrate and dichlorophenolate.

The further investigation of many of the compounds which raise the cloud point of albumin solutions leads to the segregation of the compounds into two groups: (1) compounds able to raise the cloud point greatly as well as to prevent viscosity increases during treatment of albumin with urea, and (2) compounds giving high cloud points but unable to keep the viscosity of the albumin-urea system at low values.

Several explanations may be proposed for the behavior of compounds in the second group. One would be that formation of the albumin-compound complex has not proceeded to the point where the complex is stable toward the action of 6 M urea. It may be that fewer groups of the albumin molecule interact to form the heat-resistant complex than to form the urea-resistant complex.

Another explanation, more generally applicable, would be that formation of a cloud during heating of the albumin may be slowed or prevented by the formation of a denatured albumin complex which has diminished ability to aggregate to the extent necessary to form the cloud. Certain compounds, such as dodecyl sulfate, would be expected to prevent a cloud formation by actual stabilization (at low SDS-albumin ratios) and by dispersion of the denatured albumin (at high ratios).

The compounds thus far found to belong to the first group have been mentioned previously. Examples of compounds now classed in the second group would be phenyl acetate, mandelate, acetyltryptophan, benzoate, benzenesulfonate, valeryl sulfonate, and acetate.

SUMMARY

Forty aliphatic and aromatic anions have been investigated for their ability to prevent the normal viscosity rise which occurs as an albumin-urea solution ages. The criterion for stabilization was taken to be the maintenance of a viscosity relative to solvent of 1.10 after a 24 hour period of contact between the 6 M urea and dissolved bovine albumin. Albumin-urea mixtures lacking organic salts develop a relative viscosity of 1.60 under the same experimental conditions.

Seven compounds have been shown to be stabilizers as judged by the above criterion. The relative efficacy of stabilizers has been determined in terms of the mole ratio of compound to albumin necessary to keep the relative viscosity at 1.10. The effectiveness of the stabilizers decreases in the following order: dodecyl sulfate, caprylate, 2,4-dichlorophenolate, picrate, phenyl butyrate, hexyl malonate, cinnamate, and trichloroacetate. Tentative conclusions have been drawn concerning the structure necessary for a compound to be a stabilizer.

Curves for the relative viscosity *versus* concentration of albumin are presented for experiments in which the ratios of dodecyl sulfate to albumin were maintained constant. Stabilization was apparent at all concentrations of albumin as shown by lower values of $\eta_{sp}:C$ for the solutions which contained dodecyl sulfate compared to similar solutions lacking dodecyl sulfate. An increase in ionic strength from 0.08 to 0.28 did not affect the $\eta_{sp}:C$ values. Intrinsic viscosities were estimated from the data obtained in these experiments.

Comparisons have been made concerning the effectiveness of compound in raising cloud point temperatures and in preventing viscosity increase in 6 M urea solutions.

The amorphous and crystalline bovine albumins were generously provided by Dr. J. D. Porsche of the Armour Laboratories. The painstakin technical assistance of J. Legg is gratefully acknowledged.

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THE SPECIFIC ESTERASE ACTIVITY OF TRYPSIN

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The discovery of relatively simple peptide derivatives as substrates for proteolytic enzymes has introduced a new approach to the problem of the specificity of this group of hydrolytic enzymes. Although crystalline trypsin is known to catalyze the hydrolysis of internal peptide bonds of proteins, its action toward internal peptide bonds of synthetic substrates has been demonstrated for only one case (1). Since all other synthetic substrates for trypsin, described in the extensive work of Bergmann and coworkers (2), are amides of lysine and arginine derivatives and are split enzymatically into ammonia and the corresponding amino acid derivatives, the action of trypsin toward these substrates may be more adequately described as an *amidase* activity.

In the course of an investigation of the inhibition of crystalline pancreatic proteolytic enzymes by specific low molecular weight compounds the discovery was made that crystalline trypsin is likewise a powerful catalyst for the hydrolysis of certain amino acid esters. The most specific substrates of this type are α-benzoyl-L-arginine methyl ester (BAME) and α-toluenesulfonyl-L-arginine methyl ester (TSAME). It was also found that, contrary to previous reports (3), trypsin catalyzes the amide hydrolysis of α-toluenesulfonyl-L-argininamide and does so even more rapidly than it catalyzes the hydrolysis of the classical substrate, α-benzoyl-L-argininamide. The structural specificity of these esters for trypsin is approximately analogous to that of the corresponding amides, although a certain measure of cross-reactivity of one of these (BAME) with chymotrypsin exists. Both the *esterase* and *amidase* activities are apparently mediated by the same active surface configurations of the enzyme, as is evidenced by studies of various modes of partial and complete enzyme inactivation.

The results of these kinetic studies are presented in this paper.

EXPERIMENTAL

Preparations

Enzymes—Three lots of crystalline trypsin and chymotrypsin were prepared according to the method of Kunitz and Northrop (4); activation of trypsinogen was carried out by the procedure of McDonald and Kunitz

(5).[1] Although difficulty was encountered in crystallizing trypsinogen, highly active, wholly crystalline trypsin was obtained by applying the method of McDonald and Kunitz to amorphous trypsinogen. All active enzyme preparations were recrystallized once. A portion of one preparation of trypsin was further purified by trichloroacetic acid precipitation (4).

Crystalline γ-chymotrypsin was prepared from the chymotrypsin filtrates of two preparations according to the method of Kunitz (6).

One trypsin preparation was dialyzed in the cold for 72 hours against 0.001 N sulfuric acid and was subsequently lyophilized.

Substrates—α-Benzoyl-L-argininamide hydrochloride (BAA), α-benzoyl-L-arginine methyl ester hydrochloride, α-*p*-toluenesulfonyl-L-argininamide (TSAA), and α-*p*-toluenesulfonyl-L-arginine methyl ester hydrochloride were prepared essentially according to the method of Bergmann, Fruton, and Pollok (3). A slightly improved yield of benzoylarginine was achieved by adding benzoyl chloride and 10 per cent sodium carbonate to the aqueous solution of arginine monohydrochloride at rates such that the reaction mixture was kept just alkaline to phenolphthalein. By this procedure 21.2 gm. of arginine monohydrochloride yielded 24 gm. of α-benzoylarginine.

Although BAME has not been prepared in the crystalline state, it was converted to a stable, although highly hygroscopic, glass by the following procedure: The solvent was removed *in vacuo* from a methanolic solution of approximately 8 gm. of BAME until a solid foam formed. The foam was dissolved in 30 ml. of water and the pH adjusted from 0.75 to 8.75 with 5 N NaOH. A resulting slight precipitate was filtered off and the solution reacidified to about pH 3 with 5 N HCl. The water was removed *in vacuo* and two portions of absolute methanol were added and distilled *in vacuo* to remove the last traces of water. The resulting oil was then dissolved in 60 ml. of absolute methanol and the NaCl was filtered off. The solvent was again distilled *in vacuo* and the remaining oil was heated to 100° for 10 minutes. The product is a friable, white frothy glass which was stored in a vacuum desiccator. On dry days this material could be weighed without difficulty. Although the product was not suitable for analysis, its identity was proved by conversion of a portion of it to BAA.

Carbobenzoxyglycyl-L-tyrosine ethyl ester and N-carbobenzoxy-O-acetyl-L-tyrosylglycine ethyl ester were prepared according to the method of Bergmann and Fruton (7).

ϵ-Carbobenzoxy-L-lysine methyl ester hydrochloride was prepared according to Bergmann, Zervas, and Ross (8), and carbobenzoxyglycyl-DL-phenylalanine ethyl ester was a preparation previously described (9, 10).

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[1] We are indebted to Dr. C. E. Graham of The Wilson Laboratories, Chicago, for the crude pancreatic protein precipitates used in these isolations.

Tyrosine ethyl ester hydrochloride and L-phenylalanine ethyl ester hydrochloride were prepared in the usual manner.

Glycine ethyl ester hydrochloride, ethyl butyrate, and butyl acetate were Eastman preparations and were used without purification.

Methods

Amidase Acitivity—The amidase activity of the proteolytic enzymes was measured by a modification of the Conway micro diffusion technique (11). 0.75 ml. of 2 per cent boric acid solution was substituted in the inner chamber of the Conway plate for the strong acid solution used by Conway.[2] By this means the ammonia evolved could be titrated directly and difficulties encountered in the back titration in air with dilute strong base were avoided.

The reaction mixtures used for determination of amidase activity were made by mixing equal volumes of 0.1 M substrate in phosphate buffer and of enzyme solution in phosphate buffer. In earlier runs M/15 phosphate buffer was used, but later this concentration was raised to 0.1 M to insure pH control. Because of the well known instability of trypsin in the pH range in which it is active, the enzyme was dissolved in the buffer immediately before it was added to the substrate solution.

All amidase activities were determined at 25° with shaking. 0.2 ml. samples were withdrawn at intervals for analysis and were introduced into the Conway plates. After each sample was mixed with 1 ml. of saturated K_2CO_3 solution used to volatilize the ammonia, the plates were allowed to stand for at least 1 hour before being titrated with approximately 0.01 N HCl and 1 drop of Tashiro's indicator. Thus, 0.01 ml. of acid corresponded to approximately 1 per cent hydrolysis. Since the indicator color varied with the volume of the system at the end-point, water was added to the plates in which the extent of hydrolysis was small in order to bring the final volume for all titrations close to a constant volume.

Time was measured from the time of the completion of addition of the trypsin solution to the time at which the saturated K_2CO_3 solution touched the sample. It was assumed that the enzyme was inactivated immediately by the K_2CO_3 solution and that any temperature effect due to the difference between the bath temperature and room temperature could be neglected, since the difference was not large and also since the elapsed time was never more than 1 minute. The validity of the first assumption seemed to be confirmed by the fact that the blanks were always within a few thousandths of 0.025 ml.

The horizontal burettes used for these titrations were made by drawing out the ungraduated portions of Kimble Exax 1 ml. measuring pipettes and fitting the undrawn ends with Clay-Adams pipette suction units.[3] The burettes were subsequently calibrated by weighing to within 0.1 mg. the water delivered at 0.1 ml. intervals. In no case was the error large enough to warrant the application of a correction.

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[2] This modification was suggested by Miss Elaine Elkins of this laboratory.

The accuracy of the method was tested with solutions of ammonium sulfate. With 0.05 and 0.005 N solutions of ammonium sulfate as standards, the titration values could be reproduced to within 0.3 per cent.

Preliminary experiments indicated that ammonia is not evolved by saturated K_2CO_3 solution from BAA, TSAA, trypsin, or chymotrypsin.

Blank determinations were made by placing 0.1 ml. each of substrate and enzyme solutions a short distance apart on Conway plates and by tipping the plates so that the solutions were mixed with the saturated K_2CO_3 solution before they were mixed with each other.

Esterase Acitivity—For the determination of the esterase activity precise pH control in the presence of the liberated carboxyl groups necessitated the use of such large quantities of buffer that the conventional amino acid titrations were rendered very difficult. Recourse was had to a direct electrometric titration of the liberated carboxyl groups with approximately 0.2 M NaOH with a Beckman model G pH meter as a null indicator. Low concentrations of buffer were used to render the system stable against great variations in pH but still sensitive to changes in hydrogen ion activity.

Substrate solution, buffer solution, and enough water to bring the final volume to approximately 10 ml. were mixed and equilibrated in a 25° bath. The glass electrodes were introduced and 0.2 M NaOH was added from the horizontal burette previously described until the pH rose to a value 0.2 to 0.5 unit higher than the pH selected as the null point for the determination. The enzyme solution was then added and zero time taken as the time when the pH fell to the null point value. A small increment of 0.2 M NaOH was then added and the rise in pH noted. In nearly all cases the rise in pH was less than 0.2 unit. The time was again recorded when the pH fell to the null value. This process was continued until the reaction stopped or until a sufficient portion of the reaction curve had been determined to establish the nature and rate of the reaction. The setting of the pH meter was checked against a standard buffer after each run.

In most of the experiments stirring was accomplished by means of a slow stream of nitrogen bubbled through the reaction system through a syringe needle, but in the experiments involving a high concentration of enzyme, higher temperatures, or a volatile solvent, stirring was done by hand with a small glass rod.

This method of measurement gave rise to some uncertainty as to the concentration of the substrate at the moment the zero time reading was made. This is of no consequence for reactions which follow zero order kinetics. The treatment of the data for first order reactions is illustrated in a later section of this paper.

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[3] We are indebted to Dr. W. A. Perlzweig for suggesting these useful devices.

A stock solution of enzyme was made up daily, adjusted to pH 2.5 with HCl, and kept in the refrigerator when not in use.

Nitrogen determinations on the enzyme solutions were made by the semimicro-Kjeldahl method.

Results

Benzoylargininamide

Specific Activity of Trypsin—In order to test the present method of analysis and also to characterize the preparations of trypsin used in these studies, the activity of each preparation toward BAA was determined. Measurements were made at four levels of enzyme concentration over an 8-fold range.

TABLE I

Autolysis of Trypsin at pH 7.80 As Measured by Activity toward BAA

Time	$K^* \times 10^{-3}$	Activity remaining	$K_2† \times 10^{-4}$	Calculated residual activity ($K_2 = 1.14 \times 10^{-4}$)
min.		*per cent*		*per cent*
0	7.02	100		
61½	4.09	58.1	1.17	58.6
119¾	3.00	42.7	1.12	42.3
240	1.90	27.1	1.12	26.8

* Throughout this paper K will refer to the first order reaction constant defined by $K = 2.303/t \log (C_0/(C_0 - \alpha))$, where C_0 is the initial substrate concentration and α the amount of substrate hydrolyzed in time t.

† K_2 is the second order reaction constant expressed by Kunitz and Northrop (13) as $K_2 = 1/t(1/A - 1/A_0)$. In the present calculation t is expressed in minutes and A is the per cent of the initial activity, A_0, remaining at time t.

In every case the reaction was found to follow first order kinetics strictly and for each preparation the rate constant was directly proportional to the enzyme concentration. The proteolytic coefficients, C, for all lots of trypsin prepared in this laboratory were found to fall within the limits ($C = 3.2$ to 3.8×10^{-2}) reported by previous investigators (12).

The classical studies of Kunitz and Northrop (13) on the stability of crystalline trypsin have shown that at the pH of its maximum activity rapid inactivation occurs. In the present experiments the rate of autolysis of trypsin was redetermined with BAA as substrate. The stabilizing effect of the substrate was sufficient to maintain the activity of the enzyme at a constant value during each activity determination as evidenced by the adherence of the reaction to first order kinetics. 13.6 mg. of Preparation $TIII_{1z}$ were dissolved in 5 ml. of M/15 phosphate buffer, pH 7.80. 0.5 ml. was immediately withdrawn and added to an equal volume of 0.1 M BAA in the same buffer. Subsequent samples of the enzyme solution were removed at 1, 2, and 4 hour intervals and the activity against BAA was determined.

The results given in Table I show a decrease in the first order reaction constant, K, with time. In agreement with Kunitz and Northrop (13) this has been attributed to the spontaneous inactivation of trypsin and has been expressed as a second order reaction between native and denatured trypsin. With K assumed to be proportional to activity and the remaining activities expressed as per cent of the initial activity at $t = 0$, second order reaction constants, K_2, have been calculated with the equation of Kunitz and Northrop (13). The relative constancy of K_2 together with the close agreement between calculated and observed residual activities testifies to the validity of this interpretation.

Esterase Activity

Enzymatic Nature of Reaction—The discovery of the amino acid esterase activity of trypsin was made in the course of a search for a specific low molecular weight inhibitor for trypsin.[4] It was observed that the addition of BAME to the trypsin-BAA reaction system caused a marked decrease in the rate of splitting of BAA without causing any change in the order of reaction and also caused the pH of the system to fall. Qualitative experiments with weakly buffered solutions of BAME indicated that the decrease of pH upon the addition of trypsin was due to the splitting of BAME by trypsin. In one such experiment the pH fell from 7.5 to 4.3 in 35 minutes.

At pH 8 and 25° the tryptic hydrolysis of BAME is a zero order reaction throughout about 95 per cent of its course. The results of measurements at four levels of enzyme concentration over an 8-fold range are shown in Fig. 1. A plot of the reaction rate against the weight of enzyme N in the reaction system is shown in the inset graph of Fig. 1. The deviation from linearity shown by the highest trypsin concentration in the inset plot is not considered significant, since the rate of this reaction approaches the limiting rate which can be accurately measured with the apparatus used.

The chemical nature of the reaction was established by allowing trypsin to act upon 560 mg. of BAME at pH 8 until the reaction was completed. The reaction system was dried *in vacuo* and extracted with absolute ethanol to separate benzoylarginine from the buffer salts. The ethanol was removed *in vacuo* and 339 mg. of α-benzoylarginine, corresponding to 72 per cent of the BAME used, was recovered following crystallization from water. The recovered benzoylarginine decomposed at 273–275° as did an authentic sample, and its identity was further established by the isolation of benzoic acid following alkaline hydrolysis of a portion of the recovered material.

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[4] This investigation is still in progress.

The following additional observations indicated that the hydrolysis of BAME is mediated by trypsin: (1) alkali-inactivated trypsin has no effect upon BAME; (2) the addition of increasing amounts of crystalline, salt-free soy bean trypsin inhibitor[5] progressively reduced the rate of splitting of BAME and of BAA by trypsin;[6] (3) the rate of autolysis of trypsin at pH 7.80 and 25° is the same when measured by its activity toward BAME and toward BAA. The identity of the autolysis rates of the two activities is shown in Fig. 2 in which, in analogy to the corresponding measurements of amidase activity, the tryptic activity was found to decrease with time according to second order kinetics (13). The experimental points may be

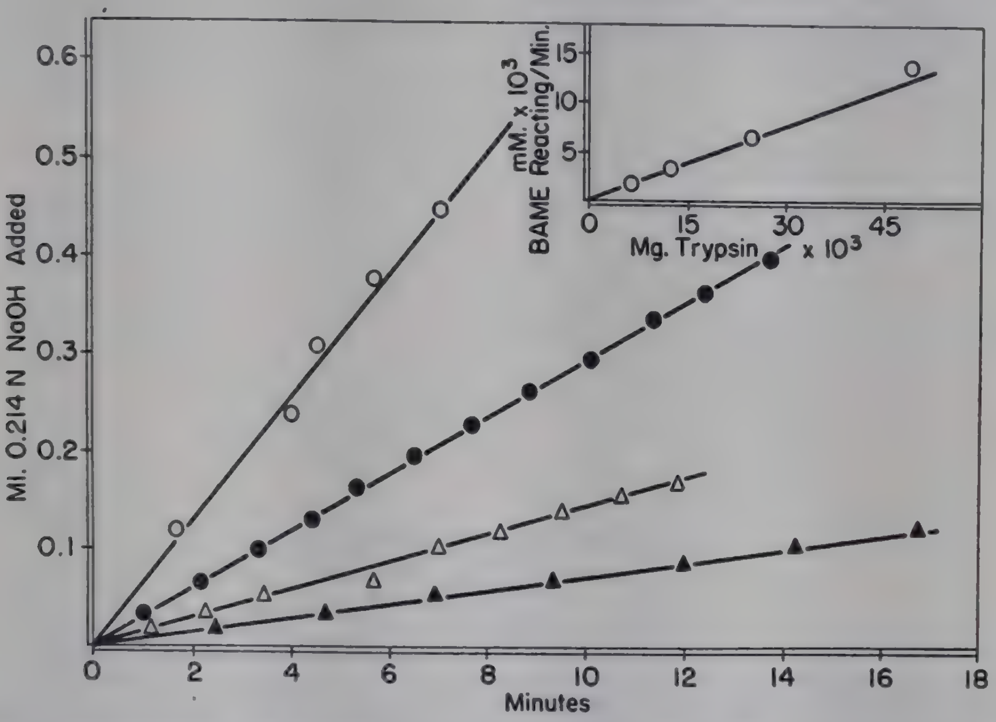

FIG. 1. Hydrolysis of BAME by various concentrations of trypsin measured by electrometric titration in 0.015 M phosphate buffer, pH 8.00, at 25°. The amount of trypsin N in mg. present in these systems is shown by ○ 0.0492, ● 0.0246, △ 0.0123, ▲ 0.0062. The inset curve indicates the linear relation between the rate of hydrolysis of BAME and the amount of trypsin present.

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[5] We are deeply grateful to Dr. M. Kunitz for two samples of crystalline trypsin and for the crystalline soy bean trypsin inhibitor used in these experiments.

[6] Work is still in progress to determine whether the linear relation between decrease in tryptic activity and amount of trypsin inhibitor added, established by Kunitz (14), using protein substrates, holds equally for low molecular weight substrates.

seen to fall on the theoretical curve calculated from the data for BAA given in Table I.

As is shown in Table II, all preparations of trypsin catalyze the hydrolysis of BAME, and the ratio of the specific reaction rate for BAME to the specific initial slope of the trypsin-catalyzed hydrolysis of BAA is essentially a constant of all preparations of trypsin tested. This method of comparing the rates of reactions of different orders was selected as offering the most valid basis for comparison, since in the initial phase of the reaction, the first order reaction, most nearly approaches a zero order reaction.[7]

Enzymatic Specificity of Reaction—The enzymatic specificity of the hydrolysis of BAME was tested at pH 8.0 with crystalline chymotrypsin, γ-chymotrypsin, chymotrypsinogen, carboxypeptidase, and ribonuclease. Of these, eight times recrystallized chymotrypsinogen (0.13 mg. of N per ml. of reaction mixture), six times recrystallized carboxypeptidase (0.009 mg. of N per ml. of reaction mixture), and ribonuclease[8] (1 mg. of crystalline enzyme per ml. of reaction mixture) had no measurable effect on 0.015 M BAME in 0.02 M phosphate buffer. In each case the subsequent addition

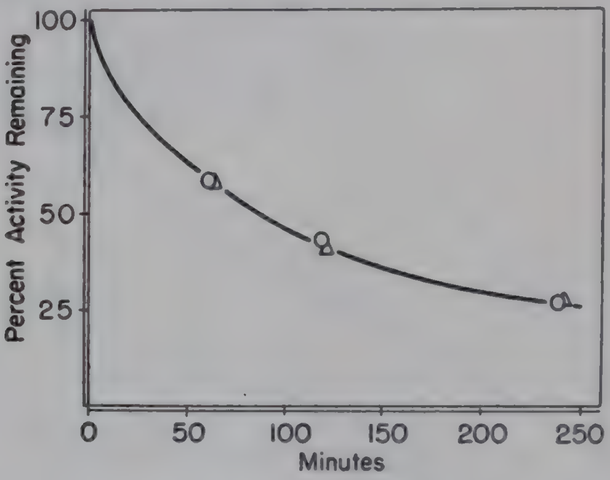

FIG. 2. Decrease in activity with time of a solution of trypsin (0.201 mg. of N per ml.) in M/15 phosphate buffer, pH 7.80, at 25°. ○ represents activity measured against BAA and △ activity measured against BAME. The curve is the theoretical curve calculated from $K_2 = 1/t(1/A - 1/A_0)$ (13) where $K_2 = 1.14 \times 10^{-4}$ (*cf.* Table I).

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[7] It would be expected that, if the difference in reaction rates between BAA and BAME were due to a difference in the rate of desorption of the products from the enzyme surface, the addition of ammonium salts to the BAME system would decrease the rate of hydrolysis of BAME. This was not found to be true. Even in 0.1 M NH_4Cl the specific hydrolysis rate of BAME remained constant.

[8] We are greatly indebted to Dr. Lawrence L. Lachat, Armour and Company, Chicago, for the ribonuclease, crystallized from alcohol, used in this experiment.

of a small amount of trypsin caused a rapid decrease in the pH of the reaction system.

Both chymotrypsin and γ-chymotrypsin, however, were found to hydrolyze BAME, the reaction following first order kinetics. The reaction curves for these hydrolyses are shown in Fig. 3 for one concentration of γ-chymotrypsin and three concentrations of chymotrypsin.

Since, by the direct titration method of measurement, the reaction starts before the zero time reading is made and since the initial concentration of BAME is not well defined, the following procedure was devised for calculating first order reaction constants. The experimental data were plotted, time being plotted on the axis of abscissas and the amount of base added on the axis of ordinates, and the best curve was drawn through the points. From the curve a series of pairs of abscissas was selected such that for each pair $t_1 = t_2/2$. If the corresponding ordinates are designated as α_1 and α_2, it follows from the properties of the first order equation, $K = (2.303/t) \log (C_0/(C_0 - \alpha))$, that $C_0 = \alpha_1^2/(2\alpha_1 - \alpha_2)$. This procedure yields a series of tentative values for C_0, the only limitation on any one value being that it must be less than the known concentration before the reaction started. With the mean value for C_0, $\log C_0/(C_0 - \alpha)$ was computed for several points and plotted against t. If these points failed to show a trend of variation from a straight line, the theoretical reaction curve was then

Table II

Hydrolysis of BAME by Various Preparations of Trypsin

Temperature 25°, 0.015 M phosphate, pH 8.00. Approximate initial concentration of BAME 0.0063 M.

Trypsin preparation	Concentration	Slope of reaction curve	K_0*	$\frac{K_{BAA} \cdot C_0}{E}$†	$\frac{K_0}{K_{BAA} \cdot C_0 \cdot E^{-1}}$
	mg. N in system	*mM per min.*	*mM per min. per mg. N*	*10^{-3} mM per min. per mg. N*	
TI_{1x}, lyophilized	0.0110	0.00274	0.249	3.92	64
$TIII_{1x}$			0.278‡	4.25	65
TIV_{1x}	0.0087	0.00212	0.244	3.56	67
CCl_3COOH purified T	0.0089	0.00212	0.238	4.16	57
Kunitz' T_{2x}	0.0124	0.00282	0.228		
" Lot 2	0.0099	0.00256	0.259		

* K_0 will be used throughout this paper to refer to specific zero order reaction constants, *i.e.* per mg. of enzyme N in the system.

† $K_{BAA} \cdot C_0/E$ expresses the initial slope of the BAA hydrolysis curve per mg. of trypsin N per ml. (E).

‡ Mean of several determinations.

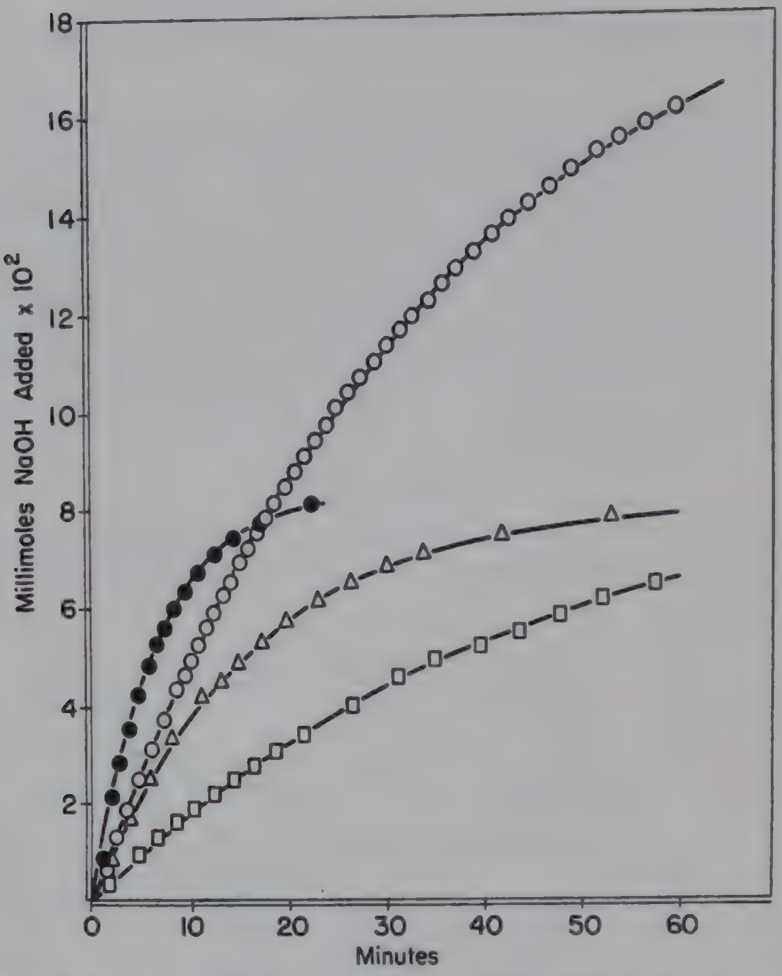

FIG. 3. The rate of hydrolysis of BAME by chymotrypsin and by γ-chymotrypsin at 25°. The curves are the theoretical first order reaction curves calculated from the following constants.

Symbol	Enzyme	Enzyme concentration, mg. N per ml.	pH	Buffer	$K \times 10^{-2}$	C_0 (calculated)
						10^{-2} *m.eq.*
○	γ-Chymotrypsin	0.0806	8.00	0.015 M phosphate	2.70	20.1
●	Chymotrypsin	0.3785	7.60	0.0185 " "	14.9	8.36
△	"	0.196	7.80	0.0185 " borate	6.37	7.98
□	"	0.0482	7.60	0.0185 " phosphate	2.22	8.92

calculated. The fit of these calculated curves to the experimental points is illustrated in Fig. 3.[9]

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[9] In several cases the tentative values of C_0 were found to increase as larger values of t_1 were selected. In these instances greater weight was given to the values of C_0 determined for larger values of t_1 because the initial points of the reaction curve were subject to a greater error in measuring times than were subsequent points. This

It may be seen qualitatively from Fig. 3 that the rate of hydrolysis of BAME by chymotrypsin increases with the enzyme concentration. The mean value for the proteolytic coefficient of chymotrypsin on BAME is 1.6×10^{-1} (C_0 of BAME about 0.01 M) and of γ-chymotrypsin (single determination) 1.5×10^{-1} (C_0 of BAME about 0.02 M). Since the corresponding mean value of $K \times C_0/E$ for chymotrypsin is 3.7×10^{-3}, it appears that trypsin splits BAME about 65 times as rapidly as does chymotrypsin (*cf.* Table II).[10]

Effect of pH—It was observed that at pH values greater than 8 BAME undergoes spontaneous hydrolysis at 25°. This phenomenon was investigated as a preliminary to the measurement of the effect of pH on the enzymatic hydrolysis of BAME. The extent of the spontaneous hydrolysis of BAME at 25° and at pH 9, 10, and 11 is shown by the continuous curve and by the initial regions of the discontinuous curves in Fig. 4.[11]

In subtracting the correction for spontaneous hydrolysis from the data obtained in the presence of trypsin, it was assumed that the correction increased linearly with time. Since the reaction in the presence of trypsin is relatively rapid compared to the spontaneous hydrolysis and since the enzymatic runs at pH 9 and 10 show no deviation from linearity except at the extreme end of the reaction, this assumption seems to be a valid first approximation.

Since the rate of the spontaneous hydrolysis is a function of the concentration of BAME (first order kinetics), while the rate of the enzymatic hydrolysis depends only upon the amount of trypsin present (zero order

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treatment neglects the dilution of the system by the base added in the course of the reaction. For a first order, non-enzymatic reaction this dilution would have no effect upon K, but for an enzymatic reaction the additional reduction in the concentration of the substrate will tend to decrease slightly the tendency for enzyme-substrate complex formation. This change will be relatively small, since the dilution in no case amounted to more than 10 per cent of the initial volume and it has, therefore, been omitted from this calculation.

[10] Because of this cross-reactivity between trypsin and chymotrypsin with BAME, the action of chymotrypsin on BAA was reinvestigated. A chymotrypsin concentration of 0.448 mg. of N per ml. of reaction mixture caused no hydrolysis of 0.05 M BAA. This observation is in agreement with the results of Bergmann, Fruton, and Pollok (3).

[11] The discontinuous curve for pH 10 of Fig. 4 is of particular interest, since the plateau reached after the addition of trypsin defines a rather small region within which the value of C_0, calculated from the initial portion of the curve, must fall if this treatment of the data is satisfactory. It will be observed that the agreement between the experimental and calculated values for C_0 is quite good and that the values for K for the two curves at pH 10 show fair agreement despite the fact that C_0 for the continuous curve has about twice the value of C_0 for the discontinuous curve (see the legend to Fig. 4).

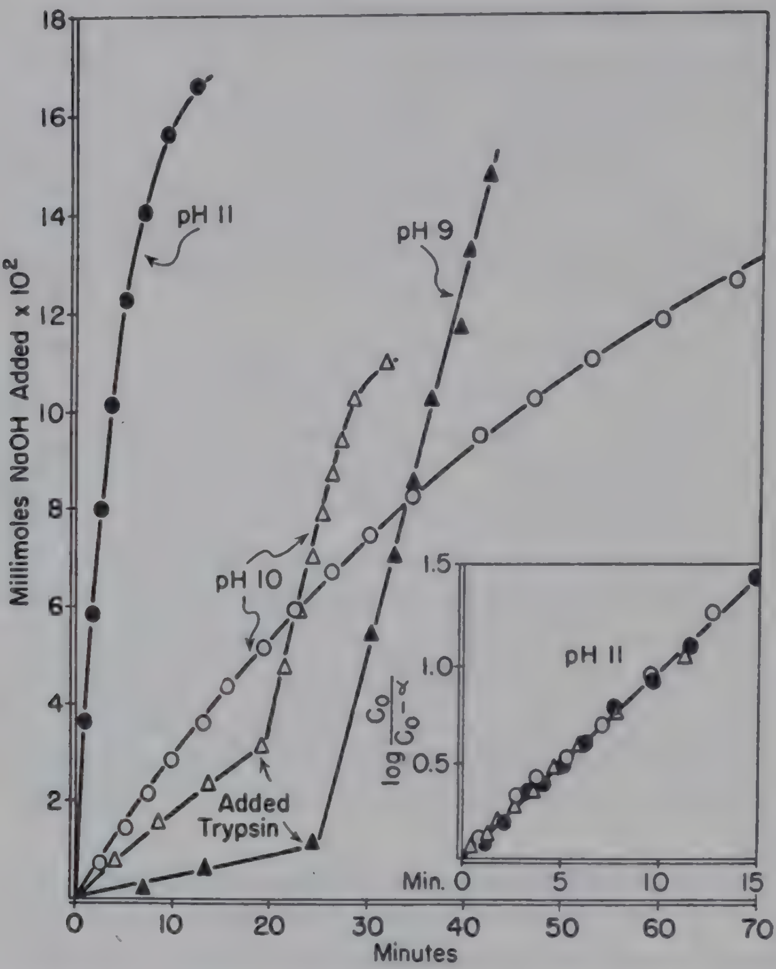

FIG. 4. Rates of hydrolysis of BAME under various conditions of pH, trypsin concentration, and BAME concentration. The curves shown for the first order reactions are the theoretical curves calculated from the values of K and C_0 shown below. Borate buffers were used in these systems.

pH	Symbol	Trypsin concentration, mg. N in system	Buffer concentration	$K \times 10^{-2}$	C_0 (calculated)
			M		*10^{-1} m.eq.*
11.00	●	0	None	22.2	1.76
10.00	○	0	0.03	1.55	1.97
10.00	△	0	0.03	1.67	1.12
10.00*	△	0.0248	0.024		
9.00	▲	0	0.03		
9.00*	▲	0.0248	0.024		

* After trypsin was added.

The inset graph illustrates the first order kinetics of the hydrolysis of BAME at pH 11 in the absence of a buffer. K for the plotted line is 2.22×10^{-1}. C_0, calculated in milliequivalents $\times 10^{-1}$, 1.76 (○), 1.70 (●), and 1.58 (△) in 0, 0, and 0.0195 mg. of trypsin N in system.

kinetics), the magnitude of the correction applied for spontaneous hydrolysis was somewhat arbitrary. It was chosen to apply to the initial concentration of BAME.

The pH-activity curve for the tryptic hydrolysis of BAME is shown in Fig. 5. The pH of maximum activity is about pH 8, the activity falling off rapidly below pH 7 and above pH 10. The rate of decrease in activity above pH 10 is intermediate between that observed with trypsin on BAA (3) and that observed with trypsin on casein (13). The inset in Fig. 4 illustrates the point that at pH 11 trypsin does not increase the rate of splitting of BAME over the rate of the spontaneous hydrolysis and that the same first order reaction constant applies to the hydrolysis of BAME in both the presence and absence of trypsin.

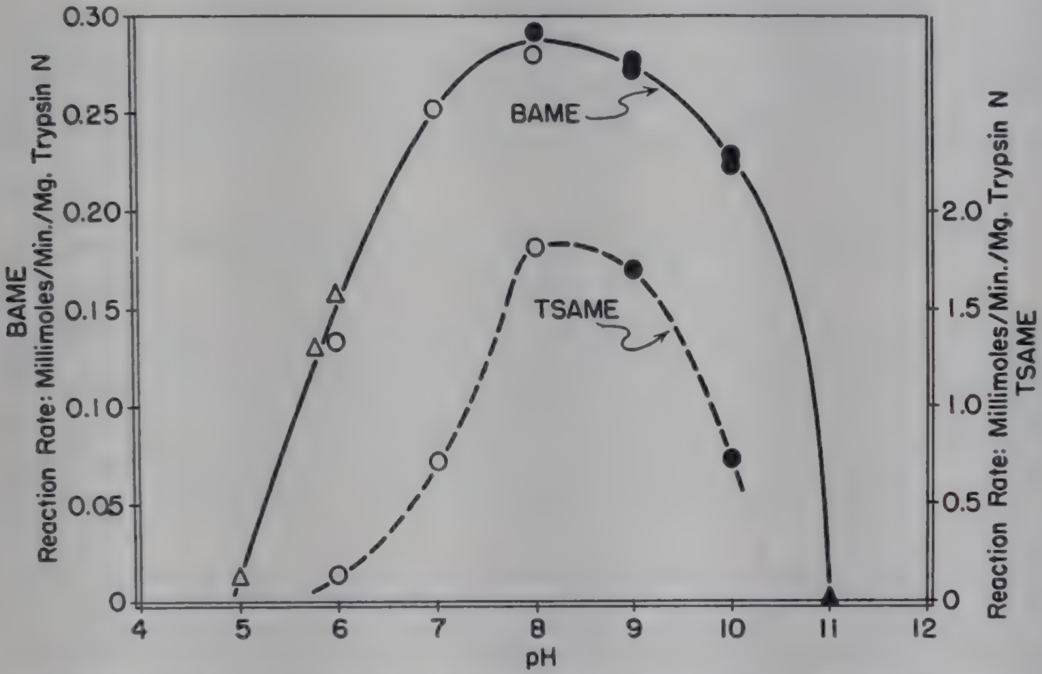

FIG. 5. Curve showing the effect of pH on the hydrolysis of BAME and TSAME by trypsin at 25°. The buffer concentration for the BAME determinations was 0.025 M and for the TSAME determinations, 0.015 M. ○ phosphate, ● borate, △ acetate, ▲ no buffer.

Effect of Temperature—The spontaneous hydrolysis of BAME was also studied at temperatures between 0.5° and 42°. Although no spontaneous hydrolysis of BAME could be observed at pH 8 at 25°, a slow but measurable reaction does occur at 42°. The spontaneous reaction was so slow at pH 10 and at pH 11 at 0.5° that only the initial slope of the reaction curve could be measured. At pH 10 at 25° and 42° and at pH 11 at 25° the reaction is clearly first order in character. At pH 11 at 42° the reaction rate is too high to be measurable by this method. From these analyses the value of Q_{10} for the spontaneous hydrolysis of BAME was found to be about 3.8.

The hydrolysis of BAME by trypsin was studied in 0.015 M phosphate buffer, pH 8.0, at 0.5°, 25°, and 42°. The reaction at 42° deviates slightly from zero order but the deviation is so small that the initial slope of this curve is probably strictly comparable to the slopes of the zero order curves measured at the lower temperatures. The corresponding reaction constants, K_0, are 0.0515, 0.273, and 0.765 (corrected for spontaneous hydrolysis) mM per minute per mg. of trypsin N. The values of Q_{10} are 1.97 (0.5–25°) and 1.83 (25–42°). These values are somewhat lower and decrease less rapidly with increasing temperature than the values determined by Butler for the action of trypsin on BAA (15). Calculation of Butler's data yields Q_{10} (6–15.2°) = 3.00, Q_{10} (15.2–25.5°) = 2.36, and Q_{10} (25.5–37.5°) = 1.82.

Substrate Specificity of Trypsin and Chymotrypsin toward Esters and Amides

The substrate specificity of trypsin and chymotrypsin was investigated with a variety of esters, amino acid esters, and peptide esters which are related to the specific substrates. Toluenesulfonylarginine methyl ester (TSAME) was found to be split by trypsin even more rapidly than is BAME. The pH activity relation for the action of trypsin on TSAME is shown in Fig. 5.

The reaction follows zero order kinetics between pH 6 and 8, but at pH 9 and 10 approaches first order. Reaction rates at the latter pH values were determined from the initial slopes of the reaction curves. TSAME was not found to undergo spontaneous hydrolysis at any pH studied. Comparison of the specific reaction constants shown in Fig. 5 indicates that trypsin hydrolyzes TSAME about 6 times as rapidly at pH 8 as it does BAME.

The results obtained with other esters are shown in Table III.

The relative insolubility of some of these substrates in aqueous solutions necessitated the use of 50 per cent ethanol for enzymatic analyses. For comparison, the activity of trypsin toward BAME in 50 per cent ethanol was determined. To 12 ml. of a 50 per cent (by volume) buffered ethanolic solution, pH 8, of approximately 0.01 M BAME was added 0.2 ml. of a trypsin solution containing 0.135 mg. of N per ml. The reaction curve deviated slightly from a zero order curve, but from the slope of the main portion of the curve K_0 was calculated to be 0.19, a value not markedly lower than that found for the tryptic hydrolysis of BAME in aqueous solutions. This is in agreement with the findings of Risley, Buffington, and Arnow (16) who studied the digestion of proteins by trypsin in alcoholic solutions.

The observation that TSAME is split by trypsin led to a reinvestigation of the splitting of toluenesulfonylargininamide by trypsin (3). Preliminary experiments, with the Conway method, have indicated that trypsin splits TSAA about 1½ times as rapidly as it splits BAA.[12] Estimates of the proteolytic coefficient toward TSAA are of the order of 6×10^{-2}. This evidence combined with the observation that chymotrypsin splits BAME

TABLE III

Effect of Trypsin and Chymotrypsin on Various Esters

Substrate	Trypsin		Chymotrypsin	
	Concentration	Rate*	Concentration	Rate*
	mg. N per ml.		*mg. N per ml.*	
L-Tyrosine ethyl ester HCl	0.0285	±		
Glycine ethyl ester HCl	0.0285	0		
Ethyl butyrate†	0.0285	0		
Butyl acetate	0.0285	0		
N-Carbobenzoxy-O-acetyl-L-tyrosylglycine ethyl ester‡	0.0363	±	0.0556	0§
L-Phenylalanine ethyl ester HCl	0.0399	±		
Carbobenzoxyglycyl-DL-phenylalanine ethyl ester‡	0.0399	0		+‖
ε-Carbobenzoxy-L-lysine methyl ester HCl‡	0.0363	0		
Carbobenzoxyglycyl-L-tyrosine ethyl ester‡	0.0399	0	0.0185	$K = 0.14$§
α-Toluenesulfonylarginine methyl ester HCl	(See text)	+	0.0705	±¶

For the water-soluble substrates the volume of the reaction system was 10 ml. Substrate concentration M/40 and buffer concentration M/66. The buffer used was phosphate, pH 8.0. The temperature was 25° for all runs.

* The rate is expressed as 0 if the pH remained steady for 15 minutes after the enzyme solution was added, and as ± if there was a fall in pH but at a rate too low to be measurable.

† Substrate concentration 0.0145 M due to limited solubility.

‡ Determination made in 50 per cent (by volume) ethanol, containing 0.02 M phosphate buffer, pH 8.00.

§ Substrate concentration 0.0156 M.

‖ Owing to the limited quantity of this substrate available, quantitative studies were not made.

¶ γ-Chymotrypsin also failed to hydrolyze TSAME at an appreciable rate.

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[12] Toluenesulfonylarginine was isolated from the enzymatic reaction mixtures of both TSAA and TSAME. The isolated samples decomposed at 247–248°. Bergmann, Fruton, and Pollok (3) report 256–257° as the decomposition point of a recrystallized sample.

rather rapidly, while it hydrolyzes TSAME only very slowly, suggests that TSAA may be a more specific substrate for trypsin than is BAA.

DISCUSSION

The experimental data presented in the preceding section appear to provide unequivocal proof for the tryptic hydrolysis of the esters of specific amino acid derivatives. Evidence for the chemical nature of the reaction is derived from the following considerations. The large release of titratable acid observed in weakly buffered reaction systems containing BAME or TSAME can only be attributed to the hydrolysis of the ester bond of these substrates. This has been confirmed by the isolation in 72 per cent yield of benzoylarginine from the hydrolysate of BAME. Although it has not been possible to characterize BAME by chemical analysis, its ready conversion into benzoylargininamide, together with the parallel results obtained with TSAME, a crystalline, chemically identified compound, leaves little doubt about the chemical identity of this substrate.

The conclusion that the hydrolysis of these esters is catalyzed by trypsin itself and by the same active surfaces which catalyze the hydrolysis of BAA is based upon the following evidence. (1) The enzymatic purity of the trypsin preparations used in this work is the same as that established by Hofmann and Bergmann (12) for a series of crystalline samples. (2) The specific zero order reaction constant, K_0, for the hydrolysis of BAME is independent of enzyme concentration and comparable values were obtained with various enzyme preparations, including one that had been subjected to precipitation by trichloroacetic acid, a procedure equivalent to several recrystallizations (4). (3) Since the specific zero order reaction constant toward BAME is approximately proportional to the proteolytic coefficient toward BAA, both measured activities appear to be properties of the same enzyme. This is further borne out by the identity of the rates of autolysis of trypsin measured by activities toward BAME and BAA (see Fig. 2). (4) When trypsin is inactivated by alkali or by the addition of soy bean inhibitor, the enzymatic activities toward BAME and BAA decrease in a parallel fashion. (5) The pH optima toward casein (13), BAA (3), BAME, and TSAME are the same. Although the pH-activity curves of trypsin toward BAME and TSAME are broader than that toward BAA, a qualitative resemblance among the three curves is apparent.

It is of significance that under standard conditions of measurement (pH 8.0, 25°, substrate concentration varying from 0.006 to 0.025 M) the esterase activity of trypsin follows a zero order reaction, whereas the amidase activity, even in higher substrate concentrations (0.05 M), follows first order reaction kinetics. Zero order reaction kinetics were observed for BAME at all pH values between pH 5 and 10, whereas with TSAME deviations toward first order reaction kinetics were noted at pH 9 and pH 10. The molecularity of the reaction is evidently the same in both reaction systems (esters and amide). If zero order kinetics are ascribed to a complete saturation of the enzyme by the substrate at all ratios of enzyme-substrate concentration that have been studied, the higher rate and lower order of reaction in the case of the esters must indicate a considerably higher affinity of the enzyme for these substrates. It is unlikely that the rate of desorption of the reaction products is the rate-limiting step, since the readsorption of the accumulating products should then decrease the reaction rate. It is more likely that the rate of activation at the enzyme surface is a controlling factor and that the activation energy for the amides is higher than that for the esters. This conclusion is corroborated by the Arrhenius activation energy calculated from the rate measurements made at various temperatures. The corresponding values for the tryptic hydrolysis of BAME and of BAA are 11,200 calories per mole and 14,900 calories per mole (from Butler's data (15)). The higher temperature coefficient for the spontaneous hydrolysis of BAME, $Q_{10} = 3.8$, compared to that for the tryptic hydrolysis, $Q_{10} = 1.9$, is in agreement with analogous data for catalyzed and non-catalyzed reactions presented by Lineweaver (17).

A certain measure of enzymatic cross-reactivity is exhibited by trypsin and chymotrypsin toward BAME. The lower affinity of chymotrypsin for BAME is evidenced by the lower rate and higher order of the reaction (first order). In contrast, this cross-reactivity does not appear when the more specific TSAME is used as a substrate.

Both the amidase and esterase activities of trypsin show a high degree of substrate specificity. Of all the esters that have been tested only BAME and TSAME exhibit a measurable rate of tryptic hydrolysis, the latter being hydrolyzed about 6 times faster than the former. Analogously, the hydrolysis of toluenesulfonylargininamide, a substrate previously reported to be resistant to tryptic hydrolysis (3), somewhat exceeds in rate that of BAA. Although, because of the different orders of reaction, precise comparison is difficult, it is evident that the activity of trypsin toward BAME is about 60 times higher than that toward BAA (see Table II).

The negative results obtained with trypsin and ε-carbobenzoxy-L-lysine methyl ester are in agreement with the findings of Hofmann and Bergmann (18) on the effect of introduction of an ε-carbobenzoxy group into an α-substituted lysinamide. The resistance of N-carbobenzoxy-O-acetyl-L-tyrosylglycine ethyl ester to chymotryptic hydrolysis is possibly caused by the introduction of the O-acetyl group. The hydrolysis of carbobenzoxyglycyl-L-tyrosine ethyl ester and of carbobenzoxyglycyl-DL-phenylalanine ethyl ester by chymotrypsin indicates that trypsin is not the only proteolytic enzyme endowed with specific amino acid esterase activity. Present evidence seems to indicate that those esters which are readily split by proteolytic enzymes are structural analogues of the typical amide substrates.

Since the present studies were limited to simple esters, it cannot be decided whether the observed esterase activity is simply related to the lower bond energy of esters as compared to amides or whether the structural details of the ester group are of essential importance. Further studies of this problem, involving the substitution of other radicals for the amide group, are in progress.

It has been suggested that in proteins certain terminal carboxyl groups form ester linkages with the hydroxyl groups of serine, threonine, or tyrosine (19). The biological significance of the esterase activity of trypsin and chymotrypsin for this and other processes remains to be evaluated.

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SUMMARY

Crystalline trypsin exhibits esterase activity toward specific esters of amino acid derivatives. Of these, α-benzoyl-L-arginine methyl ester and α-*p*-toluenesulfonyl-L-arginine methyl ester are most active, and are hydrolyzed about 60 times faster than are the corresponding amides. The catalysis of esters is mediated by the same active surface as the catalysis of the corresponding amides. Quantitative kinetic studies, including the effects of enzyme concentration, substrate concentration, pH, and temperature are reported.

Contrary to previous reports, it is found that α-*p*-toluenesulfonyl-L-argininamide is a typical substrate for trypsin and is hydrolyzed even more rapidly than is the corresponding α-benzoyl derivative.

Although BAME is also hydrolyzed by chymotrypsin, α-*p*-toluenesulfonyl-L-arginine methyl ester is not. The ethyl esters of carbobenzoxyglycyl-L-tyrosine and of carbobenzoxyglycyl-DL-phenylalanine are readily hydrolyzed by chymotrypsin, indicating that the amino acid esterase activity may be a general attribute of proteolytic enzymes.

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STUDIES IN STEROID METABOLISM

I. METHODS FOR THE ISOLATION AND QUANTITATIVE ESTIMATION OF NEUTRAL STEROIDS PRESENT IN HUMAN URINE*

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The steroid hormones in addition to their function in sex physiology have important rôles in the regulation of carbohydrate, protein, and electrolyte metabolisms. Although abnormalities involving the steroids are known to be present in certain diseases, the exact rôle of these disturbances in the etiology and manifestations of these diseases has not been established, because, hitherto, the methods which have been available have not been satisfactory for exploring the details of steroid metabolism. In the course of a comprehensive study which has been in progress in the laboratories of the Memorial Hospital since 1940 (1–5), more adequate procedures have been developed and have been utilized in the investigation of the steroids excreted in the urine of human beings in health and in sickness. The results will be reported in a series of communications, of which this, the first, describes the methods developed for the isolation of the neutral steroids from urine and for their quantitative measurement.

Subsequent reports will include the characterization of the ketosteroids isolated (6), the application of infra-red spectroscopy to their analysis and identification (7), and the descriptions of the qualitative and quantitative patterns of the steroids excreted by normal individuals and by patients with various disorders including neoplastic growth. Such comparisons have been made possible by the application of standardized procedures involving the methods herein described to the urine specimens of a large number of individuals.

EXPERIMENTAL

Collection and Preservation of Urine—The urine specimens were collected in series of 3 to 4 day periods from hospitalized patients and 6 to 10 day

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periods from out-patients of the Memorial Hospital. In many instances the urine collections from the hospitalized patients were stored in a refrigerator without preservative; those from out-patients were accumulated in carboys containing small amounts of chloroform or toluene. Occasionally larger collections from patients with rare disorders, principally of the adrenal cortex, were obtained from other institutions. With these larger collections precautions were taken to have the urine made acid to Congo red by the addition of sulfuric acid. The pH of the urine was checked routinely when the collection was received in the laboratory; nearly all of the specimens were acid.

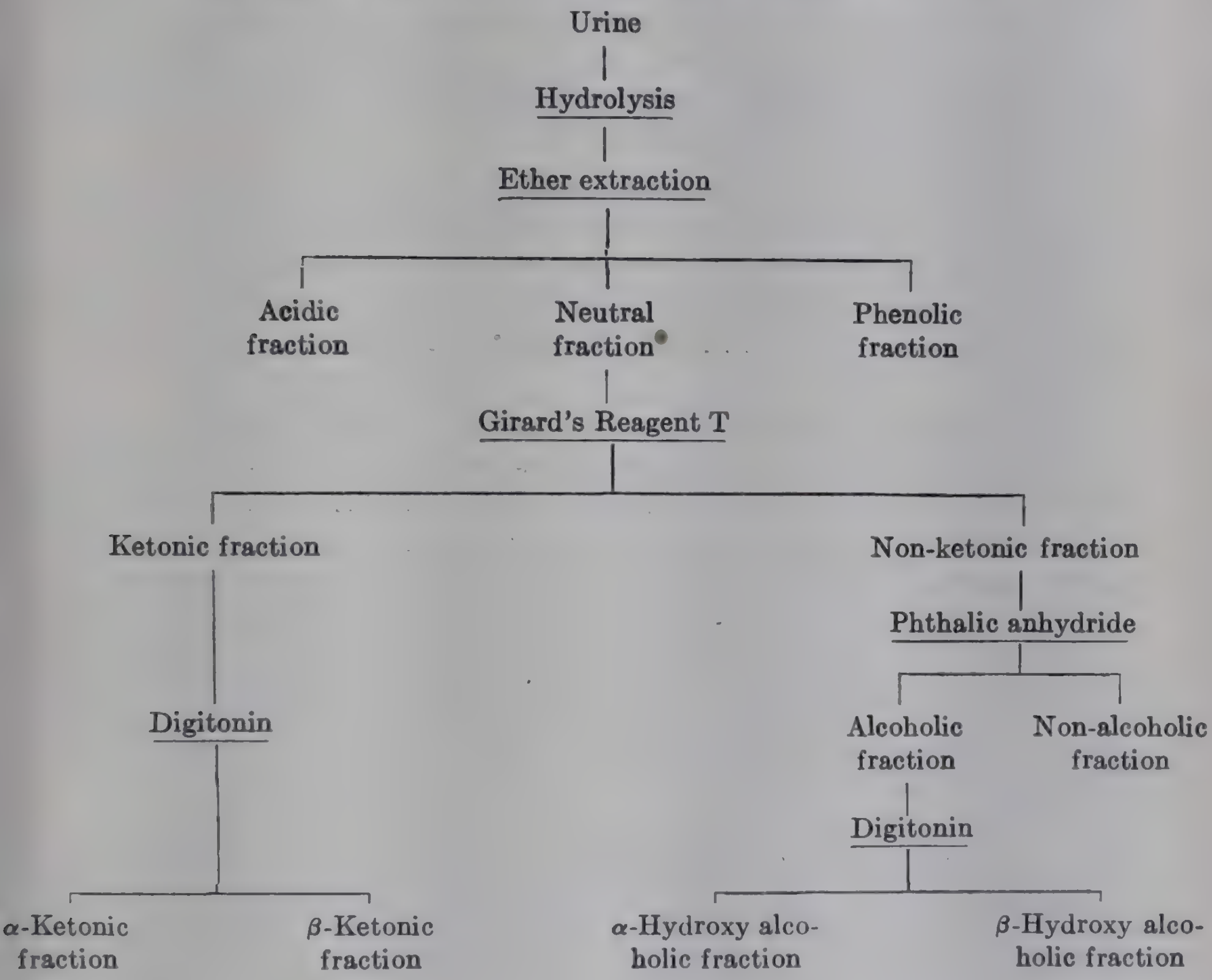
The total collection periods varied in length because of the variability in the steroid content of urine from different individuals; urines of low steroid content were collected for several months. Immediately after it was received, the urine collected in each period of the series, for example in each 3 day period, was submitted to hydrolysis, ether extraction, and separation of the ether-soluble material into the neutral, the phenolic, and the acidic fractions. These fractions were then stored until sufficient amounts of material reacting as 17-ketosteroids were accumulated. The separate fractions of the extracts of the total material collected were then combined and further purified and separated as described below. As a routine precaution the ketosteroid content of the neutral fraction was determined for the material collected during each period. The amount per 24 hours was calculated in both the long term and the 3 day collections; these usually agreed, but, when they did not, that of the short term collection was assumed to be the more accurate, and the actual number of days represented in the long term collection was calculated from the amount per 24 hours in the short term collection.

The flow sheet for the procedures employed in separating the urinary steroids is shown in the accompanying scheme. The details of the various steps are described below.

Hydrolysis of Urine—The urine was brought to pH 1 with concentrated sulfuric acid, then sufficient 50 per cent sulfuric acid was added to bring the total acidity to 5 per cent by volume, and finally the acidified solution was heated under a reflux for ½ hour.

Extraction of Urine and Fractionation of Extracts—The urine specimens after hydrolysis were extracted with ether for 24 hours in Kutscher-Steudel type continuous extractors. The ether was dispersed by passage through a fritted glass plate in order to increase the efficiency of the extraction. The extract was then concentrated to a convenient volume and separated into fractions containing the acidic, phenolic, and neutral materials by extracting the ether solution with 10 per cent sodium bicarbonate and 2 N sodium hydroxide. The sodium bicarbonate and sodium hydroxide solutions were reextracted twice with ether. The neutral ethereal solution was washed with water, evaporated to dryness under diminished pressure, and the residue was stored. At the end of each total collection period, all of the neutral fractions were combined, dissolved in ether, extracted with 2 N sodium hydroxide, and washed with water. The solvent was then removed and the residue was designated the "crude neutral fraction."

Flow Sheet for Extraction and Separation of Steroids from Urine



The acidic fraction was obtained by acidification of the sodium bicarbonate solution, followed by continuous extraction with ether. All of the individual fractions of this type from each subject were combined to give the total acidic fraction. The solvent was removed and the residue saved for later investigation. The phenolic fraction was similarly obtained from the 2 N sodium hydroxide solution and stored for future work.

Analytical Procedures

Quantitative Determination of Ketosteroid Content by Zimmermann Reaction—The ketosteroid content of the neutral fraction was measured by the Zimmermann reaction with use of the Callow absolute alcohol technique (8, 9) as modified by Talbot *et al.* (10). This procedure was used in preference to the determination of the weight of the total solids, since many of the fractions contained large amounts of substances other than steroids. In colored samples, corrections for blank readings were made, but no other color correction factors were used (10, 11). Androsterone was used as the standard reference compound, and hence all ketosteroid values are expressed in mg.-equivalents of androsterone.

As purification of the neutral fractions proceeded, the color obtained by the Zimmermann reaction became more typical of that given by androsterone. After chromatographic separation, the highly purified 17-ketosteroids showed the characteristic color given by androsterone, while the 20-ketosteroids gave atypical colors characterized by much lower extinction coefficients (12, 13).

The average daily ketosteroid value (Column 7, Table I) for a long collection period was calculated by dividing the total ketosteroid value of the "crude neutral fraction" by the number of days of collection. A check on this value was obtained by the procedure previously described. The ketosteroid contents of the various fractions, *e.g.* α-ketonic (ketosteroids not precipitated by digitonin), β-ketonic (ketosteroids precipitated by digitonin), etc., were similarly determined by the Zimmermann reaction, and the values were computed in terms of the average daily excretion. The results obtained in a representative series of normal and abnormal individuals are given in Table I.

Quantitative Determination of Alcoholic Groups in Non-Ketonic Steroids—The method used to determine the hydroxyl groups of the steroids in the non-ketonic fractions depends on the formation of the half acid phthalate esters and the titration of these with dilute alkali (14). Since indicators could not be used because of the color present, the titration was followed electrometrically by means of the Coleman glass electrode.

A weighed aliquot of the alcoholic fraction containing from 5 to 30 mg. of material was taken for analysis. The sample was transferred to a small, long neck flask with a 24/40 standard tapered joint, benzene was added, and the material dried by distillation of the solvent, followed by evacuation. 1 ml. of a pyridine solution of phthalic anhydride which contained about 30 mg. of anhydride was introduced into the flask, an air condenser protected by a calcium chloride tube was attached, and the solution was heated in an oil bath at 130° for 3 hours. At the end of this period, 5 ml. of water were added and the mixture was allowed to stand at room tempera-

TABLE I

Values Obtained in Fractionation of Crude Neutral Extracts from Urine of Normal Subjects and Diseased Persons

Subjects				Collection		Amount per 24 hrs.							
							Ketonic fraction, color equivalent[*]			Non-ketonic fraction			
											Alcoholic fraction		
Case No. (1)	Diagnosis (2)	Sex (3)	Age (4)	Total volume of urine (5)	No. of days (6)	Total crude neutral extract color equivalent[*] (7)	Total (8)	α (9)	β (10)	Total weight (11)	(12)	(13)	Non-alcoholic fraction (14)
			yrs.	*liters*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*microgram-equivalents*	*mg.*
NM1	Normal	M.	21	168	114	17.1	16.7	16.2	1.1	44.6	10.8	34.2	32.0
NM2II	"	"	35	91	33	29.0	26.1	21.4	2.3	99.0	21.2	41.0	72.5
NF1	"	F.	17	140	141	15.8	16.2	13.6	0.4	34.4	6.4	28.3	30.0
NF2I	"	"	32	86	42	14.7	14.9	10.1	0.5	61.4	7.9	45.0	57.6
P1	Pregnancy	"	33	117	171	7.0	4.5	4.2	0.1	54.2	15.6	78.0	36.4
AHF1	Adrenal hyperplasia	"	8	134	95	62.0	65.8	62.3	3.2	135.9	50.2	188.4	84.0
AHF2	" "	"	27	20	40	53.7	54.6	43.7	3.7	89.8	30.0	121.0	59.7
ATF1	" tumor	"	19	4	14	192.8	212.1	130.7	80.6	258.4	72.6	362.0	164.9
ATF2	" "	"	28	17	13	71.0	41.7	30.1	11.0	129.7	38.9	195.0	86.2
CaBF1	Cancer of breast	"	62	396	223	8.6	6.4	6.3	0.1	35.5	5.7	28.0	27.3
CaBF2	" " "	"	33	117	249	6.3	6.1	5.5	0.4	26.2	5.3	24.8	20.0
CaP1	" " prostate	M.	60	271	152	6.9	3.6	3.5	0.2	51.0	5.4	22.4	46.6
CaP2	" " "	"	65	188	200	6.6	2.8	2.6	0.01	66.0	5.3	15.3	56.9

Note that, although theoretically the value of Column 7 is equal to the sum of the values of Columns 8, 12, and 14, it is not possible to perform this calculation because the values in Columns 7 and 8 are measured by color equivalents in terms of androsterone as a standard, while the values in Columns 12 and 14 are measured by weight. Furthermore, while the value of Column 8 approximates the sum of the values of the Columns 9 and 10, and the value of Column 11 approximates the sum of the values of Columns 12 and 14, there are minor discrepancies due to either an elimination of chromogenic or other non-steroid material or to a slight loss of steroid during the fractionation procedures. For further discussion see the text.

[*] In terms of mg. of androsterone as a standard.

ture for 10 minutes. The solution was then transferred quantitatively to a beaker by washing first with 25 ml. of ethanol and then with 20 ml. of water. The electrodes, stirrer, and burette tip were placed in this beaker below the surface of the solution. The titration was carried out with 0.02 to 0.03 N sodium hydroxide. The end-point was determined by the graphic method (15).

The microgram-equivalents in the alcoholic fraction were calculated by the following expression.

Microgram-equivalents = 1000 × N × ($\text{ml.}_a - \text{ml.}_b$)
where N = normality of the alkali used
ml._a = ml. of alkali consumed in the titration of 1 ml. of the pyridine solution of phthalic anhydride
ml._b = ml. of alkali consumed in the titration of the unknown sample

Since ($\text{ml.}_a - \text{ml.}_b$) is a measure of the free carboxyl groups in the phthalate half esters, it is proportional to the amount of alcoholic steroid present.

The procedure was applied to several pure compounds (Table II). It is apparent that not only an OH group at C_3 of the steroid nucleus forms a half ester with phthalic acid, but also the C_{17} and C_{20} OH groups, in androstanediol-3α,17α and pregnanediol-3α,20α, respectively, likewise react to form half esters under the conditions of the determination.

The values obtained on representative non-ketonic alcoholic fractions from urine are given in Table I (Columns 12 and 13). It is important to note that the values represent a measure of the hydroxyl groups capable of reacting with phthalic anhydride under the specific conditions of the determination; the magnitude of the value depends not only on the absolute amount of steroid present but also on the number of reactive hydroxyl groups present in the various constituent steroids. Thus, the higher value (Table I, Column 13) for microgram-equivalents of the non-ketonic alcoholic fraction of pregnancy urine (as compared with the same fraction from normal urine) is in part due to the higher content of pregnanediol with two reactive hydroxyl groups.

Application of Ultraviolet and Infra-Red Spectrometry—The application of ultraviolet spectrometry to steroids is limited to the detection of conjugated unsaturated groupings. Standard technique with the Beckman quartz spectrophotometer has been employed. Infra-red spectrometry has been used extensively in these investigations, and the experience with this procedure is the subject of a separate communication (7).

Neutral Fraction Separation Procedures

Separation of Ketones from Non-Ketones by Girard's Reagent T—The ketonic and non-ketonic constituents of the "crude neutral fraction" were separated by means of Girard's Reagent T (16) in the usual manner with 10 ml. of absolute ethanol, 1.25 ml. of glacial acetic acid, and 1.0 gm. of the reagent for each 100 mg. of ketosteroid present. The ketones were recovered from the hydrazones by acid hydrolysis at pH 1 at room temperature for at least 16 hours, followed by continuous extraction of the hydrolysates with ether for 24 hours. The hydrolysis of certain steroid hydrazones is not always complete in a period shorter than 16 hours at pH 1 and the exhaustive extraction with ether is necessary to effect complete recovery of certain highly oxygenated water-soluble steroids. The 17-ketosteroid contents of the resulting ketonic and non-ketonic fractions were determined routinely, although the non-ketonic fractions invariably gave atypical brown colors. The ketosteroid values of the ketonic fraction for a representative group of individuals are given in Table I (Column 8).

TABLE II

Determination of Pure Alcoholic Steroids by Formation and Titration of Acid Phthalate Esters

Substance	Weight	Refluxing time	Found*	Calculated	Yield
	mg.	*hrs.*	*microgram-equivalents*	*microgram-equivalents*	*per cent*
Androsterone	10.02	2	34.1	34.5	99
Etiocholanol-3α-one-17	10.73	2	34.9	37.0	94
Dehydroisoandrosterone	30.29	1	101.0	105.0	96
	10.21	3	35.0	35.5	99
Pregnanol-3α-one-20	10.27	1	32.9	32.3	102
	20.00	3	60.5	62.9	96
Cholesterol	24.80	1	62.9	64.3	98
	10.40	3	25.0	26.9	93
Androstanediol-3α, 17α	9.20	1	62.4	63.0†	99
	10.90	3	73.5	74.6†	98
Pregnanediol-3α, 20α	25.47	1	135.9	159.2†	86
	25.41	3	156.0	158.8†	99

* Titrated with standard 0.02 N NaOH.

† Calculated for 2 equivalents of acid.

Frequently the Girard fractionation was repeated on both the ketonic and non-ketonic materials, since it is known that such separations are not strictly quantitative. A more effective separation of the residual non-ketonic impurities is obtained by repetition of the Girard separation after a preliminary chromatographic purification.

Separation of Ketonic Steroids by Precipitation with Digitonin—The methods which have been employed by various investigators for the separation of the α- and β-ketosteroids differ principally in the concentration of aqueous ethanol used (17–19). Since Noller (20) has shown that the digitonides of different steroids vary considerably in their solubility in alcohol, it appeared preferable to choose a standard procedure in which the ratios of alcohol, water, digitonin, and ketosteroid concentrations were constant. The procedure of Butler and Marrian (21) was employed with minor modifications. The digitonides were precipitated from a 90 per cent ethanol solution in which the concentration of ketosteroid was 1 per cent, and that of the digitonin was 1.2 per cent. The amounts of α- and β-ketosteroids so separated were determined by the Zimmermann reaction. The results of such determinations for a representative group of individuals are given in Table I (α, Column 9; β, Column 10).

Separation of Alcoholic and Non-Alcoholic Non-Ketones with Phthalic Anhydride—Attempts to separate the constituents of the non-ketonic fraction by chromatographic analysis met with little success because of the relatively large amounts of non-steroidal material. When, however, the alcoholic substances were separated from the non-alcoholic components, it was possible to achieve extensive purification of the alcoholic fraction. The separation was made according to the following procedure: The thoroughly dried non-ketonic residue obtained from the Girard separation was dissolved in anhydrous pyridine, 1 ml. for every 100 mg. of residue. Freshly fused phthalic anhydride equal in weight to the non-ketonic residue was added, and the mixture heated on an oil bath at 130° for 3 hours under a reflux condenser protected with a calcium chloride tube. The reaction mixture was poured into an ice and water mixture and extracted three times with ethyl acetate. The extracts were washed with 10 per cent sulfuric acid and the acid washings were discarded. The ethyl acetate solution was extracted with six separate portions of 2 N sodium hydroxide, and then the ethyl acetate solution was washed several times with water. The ethyl acetate was removed by distillation under diminished pressure, and the residue[1] was designated the "non-alcoholic fraction."

The combined sodium hydroxide extracts were acidified to Congo red with 10 per cent sulfuric acid and extracted with three separate portions of ethyl acetate. The ethyl acetate solution was washed five times with water and the solvent was removed under reduced pressure. The residue, which contains the phthalic half esters of the alcohols and some free phthalic acid, was converted to the free alcohols by the method of Chibnall (22). It was weighed and dissolved in benzene and absolute ethanol, 5 ml. of benzene and 1 ml. of absolute ethanol being used for each 100 mg. of solid. The solution was heated to boiling, 1.5 ml. of a hot 10 per cent sodium methylate solution were added, and the heating was continued under a reflux for 10 minutes with frequent agitation. A copious precipitate of sodium methyl phthalate formed. The mixture was cooled to room temperature, an excess of water was added, and the mixture was extracted with three portions of ethyl acetate. The ethyl acetate extracts were combined and washed several times with 2 N sodium hydroxide and with water. After drying over sodium sulfate, the ethyl acetate was removed under diminished pressure, and the residue was weighed and designated the "alcoholic fraction." The results obtained when this procedure was applied to pure steroids and to urinary extracts are presented in Table III and in Table I (Column 12) respectively.

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[1] The resulting product contains acidic material which cannot be removed by repeated extraction of the ethyl acetate solution with alkali. However, it can be readily removed by dissolving the residue in carbon tetrachloride and extracting with four to six portions of 2 N sodium hydroxide.

Table III

Recovery of Crystalline Steroids by Phthalic Anhydride Separation Procedure

Substance	Weight			Titration		
	Taken	Recovered		Taken*	Recovered*	
	mg.	*mg.*	*per cent*	*microgram-equivalents*	*microgram-equivalents*	*per cent*
Dehydroisoandrosterone......	200.1	209.9	105	688	747	109
Pregnanediol-3α,20α..........	130.2	134.2	103	876	804	92
Androstanediol-3α,17α........	163.7	170.6	104	1097	1137	104

* These values are the averages calculated from at least three determinations made on accurately weighed aliquots of approximately 10 mg. each.

The microgram-equivalents of the "alcoholic fractions" (Table I, Column 13) were determined by the method previously described. The microgram-equivalents of the "non-alcoholic fractions" invariably showed a negligible content of alcoholic compounds at this stage.

Separation of Ketosteroids by Chromatographic Adsorption—The ketonic fractions were submitted to systematic chromatographic separation modeled on the procedure used in fractional crystallization. In the course of 4 years of experience it has been possible to introduce certain improvements into the original procedure, which have decreased the number of chromatograms necessary, increased the efficiency of each, and minimized the destruction of certain compounds. The general procedure which follows can be modified in accordance with the varying amount of steroids, pigments, oils, and other substances present. In general, the ketonic fractions from urine samples with a high ketosteroid content are more readily purified than those from samples with a low ketosteroid content.

Adsorbents and Solvents—Magnesium silicate (No. 34 obtained from the Philadelphia Quartz Company, Berkeley, California) diluted with 1 part by weight of Celite (analytical filter aid from Johns-Manville, New York) were used. Aluminum oxide (standardized according to Brockmann from Merck and Company, Rahway, New Jersey) was also employed. To avoid variability in quality of different lots, a large quantity of aluminum oxide

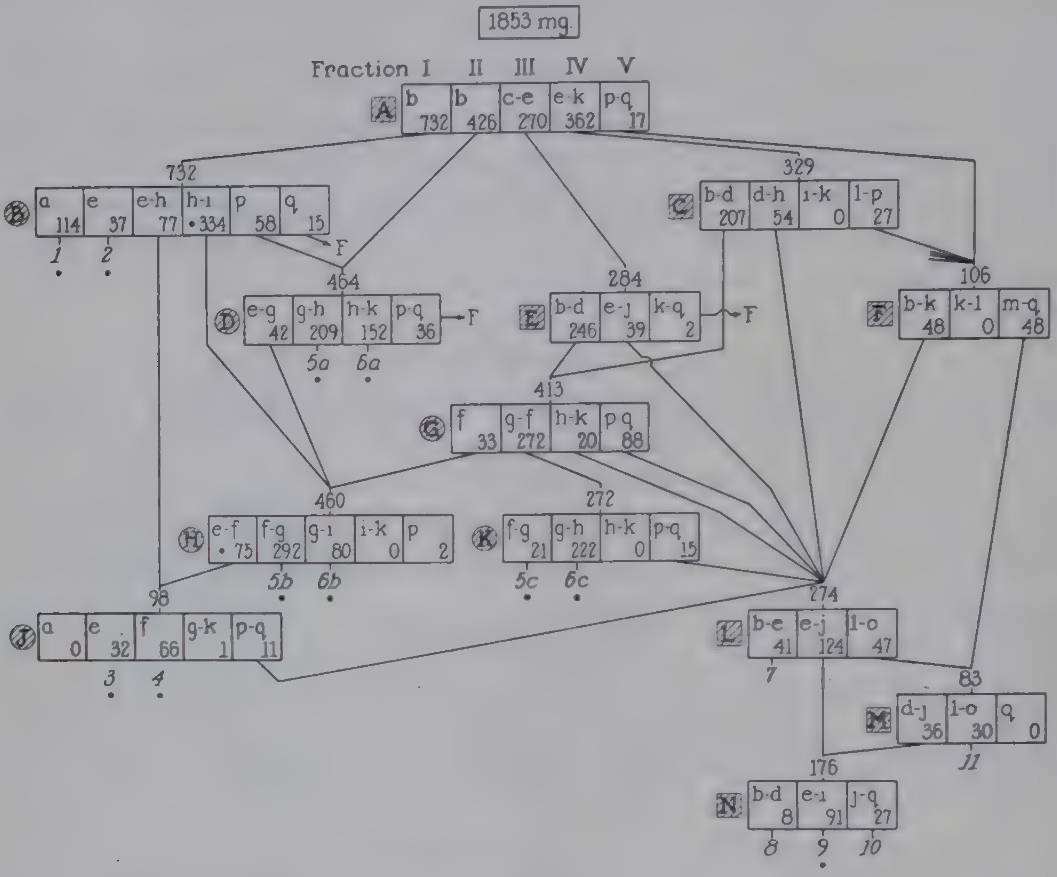

FIG. 1. Flow-sheet for systematic fractional chromatographic separation of α-ketonic steroids of urine. *a*, ligroin-benzene (1:1); *b*, CCl_4; *c*, CCl_4-benzene (1:3); *d*, CCl_4-benzene (1:1); *e*, benzene; *f*, ether-benzene (1:9); *g*, ether-benzene (1.5:8.5); *h*, ether-benzene (1:4); *i*, ether-benzene (1:3); *j*, ether-benzene (1:1); *k*, ether; *l*, methyl alcohol-ether (1:49); *m*, methyl alcohol-ether (1:19); *n*, methyl alcohol-ether (1:9); *o*, methyl alcohol-ether (1:1); *p*, methyl alcohol; and *q*, glacial acetic acid. The cross-hatched squares indicate magnesium silicate adsorbent; cross-hatched circles, aluminum oxide adsorbent; ●, crystalline fraction. The capital letters refer to Chromatograms A, B, C, etc. The amounts of ketosteroids are expressed in mg. of androsterone equivalents.

of Lot 41132 was purchased and has been used throughout the investigations.

The solvents were of C.P. or reagent grade from commercial sources. Only the ligroin was further purified by washing with concentrated sulfuric acid, 2 N sodium hydroxide solution, and water. It was then dried and distilled from calcium chloride.

Chromatographic Procedure—When magnesium silicate was used as the adsorbent, short, broad tubes (15 to 75 mm. inner diameter × 30 cm. in height) which had fritted glass plates sealed in just above the stop-cocks were found to be most satisfactory. The weight of magnesium silicate-Celite mixture used was from 60 to 150 times the ketosteroid content as determined by the Zimmermann reaction. The height of the column generally was from 1 to 3 times its diameter. A thin slurry of the finely powdered magnesium silicate-Celite mixture was prepared with carbon tetrachloride or benzene and poured rapidly into the tube. The mixture was stirred vigorously with a glass rod and then nitrogen gas under pressure from a cylinder was applied. When most of the solvent had been removed, a layer of glass wool was placed on the surface. Under no circumstances was the column permitted to become dry.

For chromatographic separation on aluminum oxide, adsorption columns from 12 to 18 mm. in diameter and 500 to 600 mm. in height with a bulb of about 300 ml. capacity fused to the top and a No-Lub stop-cock at the base were used. Plugs of absorbent cotton were inserted, the tube filled with the appropriate solvent, and the aluminum oxide poured slowly into the solvent while the tube was tapped gently with a soft rubber mallet. The stop-cock was then opened and, as the solvent flowed out, the aluminum oxide settled uniformly. The amount of aluminum oxide used was 60 to 100 times the ketosteroid content, determined by the Zimmermann reaction, present in the fraction to be chromatographed. The height of the aluminum oxide column was from 20 to 30 times the diameter.

The material to be chromatographed was dissolved in an appropriate solvent (ligroin-benzene (1:1) mixture, carbon tetrachloride, or benzene) and the concentration adjusted so that the ketosteroid content was from 2 to 5 per cent. The solution was forced through the column under nitrogen pressure and the various eluates were collected in the same manner in 50 or 75 ml. portions. The chromatogram was developed by employing a series of solvents of increasing polarity in the following sequence: ligroin, carbon tetrachloride, benzene, ether, acetone, methanol, ethanol, and acetic acid. Transition from one solvent to the following eluant was made by using mixtures of the two in varying proportions. Acetone, while valuable with magnesium silicate columns, is to be avoided with aluminum oxide columns because oily condensation products are formed which interfere with subsequent crystallization of the steroids. Elution with a particular solvent mixture was continued until no further material was removed from the column before the next solvent or solvent mixture in the series was employed. The eluates were evaporated to dryness on a steam bath, and the ketosteroid content of each routinely determined by the Zimmermann reaction. 100 to 260 eluates were obtained from a typical chromatogram.

Fractional Chromatographic Separation—As an example of the procedure employed, a short description is given of the fractional chromatographic separation of the α-ketonic steroids from the urine of a normal man (Case NM1, Table I). The urine specimens were collected for a period of 114 days and amounted to 168 liters.

A flow sheet of the chromatograms used in this separation is shown in Fig. 1. The ketosteroid content by the Zimmermann reaction of the crude α-ketonic fraction was 1853 mg. in terms of androsterone equivalents.

It is important to note that the ketosteroid values expressed as androsterone equivalents do not by any means represent either the total weight of the material or the true ketosteroid content because of the variation in chromogenic value of the different 17-keto, 20-keto, and other ketonic substances present in the extracts. The expression "mg. of ketosteroid content" is, therefore, to be understood in this particular sense, and represents a convenient index of the degree of purification or a basis for comparison with other extracts rather than an absolute measure of the individual components of the mixture.

The first chromatogram (A, Fig. 1) was carried out with 110 gm. of magnesium silicate-Celite mixture and gave a rough separation into five fractions. The first fraction (AI), eluted with 450 ml. of carbon tetrachloride, had a ketosteroid content of 732 mg. The second fraction (AII), eluted with 370 ml. of carbon tetrachloride, had a ketosteroid content of 426 mg. In the third fraction (AIII), 300 ml. of benzene-carbon tetrachloride mixture (1:3) and 300 ml. of benzene-carbon tetrachloride (1:1) yielded 270 mg. The fourth fraction (AIV), eluted with 450 ml. of benzene, 450 ml. of ether-benzene mixture (1:1), and 450 ml. of ether, contained 362 mg. The fifth fraction (AV) was eluted with 450 ml. of methanol and 300 ml. of glacial acetic acid. This highly pigmented fraction had a ketosteroid content of only 17 mg. A total of 1426 mg. of ketosteroids was thus recovered from the 1853 mg. originally determined. The difference between these two values does not indicate an actual loss of 427 mg.; rather, the discrepancy is due in part to elimination of non-specific chromogens in the course of the purification, and in part to the accumulation of errors from the numerous ketosteroid determinations.

Because separation with Girard's Reagent T is more efficient after an initial chromatogram, each of these five fractions was again submitted to a Girard separation in order to remove the non-ketonic impurities still present. For example, indigo red, present in relatively large amounts in Fraction AIII could now be removed almost completely from the ketonic fraction by the Girard separation, whereas considerably less could be removed by repeated treatment of the crude neutral material with Girard's reagent prior to chromatography. The use of the Girard separation before *each* of the subsequent chromatograms was extremely valuable, since it reduced to half the number of chromatograms necessary to achieve satisfactory separation and purification.

Fractions AI and AII were chromatographed on alumina (Chromatograms B and D). Chromatogram B was started with 1200 ml. of ligroin-benzene mixture (1:1) and continued with 900 ml. of benzene, 900 ml. of ether-benzene mixture (1:9), 1500 ml. of ether-benzene mixture (1:5), 1200 ml. of ether-benzene mixture (1:4), 300 ml. of methanol, and finally 150 ml. of glacial acetic acid. The material present in the benzene-ligroin eluate (Fraction BI) showed absorption in the ultraviolet region which indicated the presence of material containing a conjugated diene system similar to that in $\Delta^{3,5}$-androstadienone-17. From the benzene fraction (BII) crystalline material melting at 176° was obtained. The third fraction (BIII) (ether-benzene 1:9) was non-crystalline and was rechromatographed in Chromatogram J. The fourth fraction (BIV) (ether-benzene 1:5 and 1:4) contained impure androsterone which was rechromatographed in Chromatogram H. The methanol fractions (BV) were combined with Fraction AII and rechromatographed in Chromatogram D. The last fraction, eluted with acetic acid (Fraction BVI), was rechromatographed in Chromatogram F.

Fraction AIV was rechromatographed (Chromatogram C) on the magnesium silicate-Celite mixture. The first eluate was obtained with 600 ml. of benzene-carbon tetrachloride mixture (1:1) (Fraction CI); subsequent fractions were obtained with 300 ml. of benzene, 450 ml. of ether-benzene mixture (1:9), 450 ml. of ether-benzene mixture (1:4) (Fraction CII); with 300 ml. of ether-benzene (3:7) and 300 ml. of ether (Fraction CIII); and 300 ml. of methanol (Fraction CIV). None of the fractions yielded crystalline material. Each fraction was combined with similar fractions from the other chromatograms, fractionated with Girard's Reagent T, and rechromatographed as shown in Fig. 1. In this manner a nearly quantitative separation of the steroids was achieved. The sharp separation obtained is illustrated in Table IV, which shows in detail Chromatogram H, in which the diastereoisomers, androsterone and etiocholanol-3α-one-17, were separated.

Results of Chromatographic Separation—The eluates have been divided arbitrarily into three groups. The first (No. I), "early" group, is eluted before androsterone. The second (Group II), "middle" group, consists of androsterone and etiocholanol-3α-one-17. The third (Group III), "late" group, represents substances eluted after etiocholanolone. Table V gives a summary of the results of the fractional chromatographic analysis of the α-ketonic steroids of Case NM1. It shows the type and amount of

TABLE IV

Chromatogram H (Cf. Fig. 1), 460 Mg.; Ketosteroids Chromatographed on 70 Gm. of Aluminum Oxide*

Fraction No.	Eluent	Volume of eluent	Ketosteroid recovered	M.p.	Remarks
		ml.	*mg.**	°*C.*	
1	Benzene	150			Fraction HI; rechromatographed in J (*cf.* Fig. 1)
2	"	150	3.7		
3	"	300	15.5		
4	"	300	19.8	160–165	
5	"	300	4.9	Oily	
6	"	300	1.5	"	
7	Ether-benzene 1:9	300	15.1	"	
8	" 1:9	300	13.1	"	
9	" 1:9	300	1.9		
10a	" 1:9	75	2.7	177–184	Fraction HII; androsterone, 292 mg. (*cf.* Table V, Fraction 5b)
b	" 1:9	75	3.8	178–183	
c	" 1:9	75	5.0	178–183	
d	" 1:9	75	8.0	178–183	
11	" 1:9	4 × 75	36.9	178–183	
12	" 1.5:8.5	4 × 75	67.3	178–183	
13	" 1.5:8.5	4 × 75	48.7	178–183	
14	" 1.5:8.5	4 × 75	36.1	178–183	
15	" 1.5:8.5	4 × 75	29.0	178–183	
16	" 1.5:8.5	4 × 75	22.3	178–183	
17	" 1.5:8.5	4 × 75	15.7	178–183	
18	" 1.5:8.5	4 × 75	11.5	178–183	
19a	" 1.5:8.5	75	2.4	178–180	
b	" 1.5:8.5	75	2.5	180–182	
c	" 1.5:8.5	75	2.7	136–146	Fraction HIII; etiocholanol-3α-one-17, 80 mg. (*cf.* Table V, Fraction 6b)
d	" 1.5:8.5	75	2.7	136–143	
20	" 1.5:8.5	4 × 75	8.1	136–143	
21	" 1:4	300	9.9	136–143	
22	" 1:4	300	10.1	136–143	
23	" 1:4	300	9.1	136–143	
24	" 1:4	300	8.5	136–143	
25	" 1:3	300	8.4	136–143	
26	" 1:3	300	17.4	143–146	
27	" 1:3	300	2.9		
28	" 3:7	300			Fraction HIV
29	" 3:7	300			
30	" 2:3	300			
31	" 2:3	300			
32	" 1:1	300			
33	Ether	300			
34	Methyl alcohol	300	2.4		Fraction HV
Total			449.6		

* Androsterone equivalent in the Zimmermann reaction.

TABLE V

Summary of Fractions Obtained from Systematic Chromatographic Separation of α-Ketonic Steroids (See Fig. 1)

Chromatogram	Fraction No.	Amount*	Nature of fraction	
		Group I ("early")		
		mg.		
B	1 and 2	129	M.p. 108–110°; mixture of $\Delta^{3,5}$-androstadienone-17 and Δ^{2}-androstenone-17 and gum	
" J	2 " 3	46	M.p. 125–155° and gum	
J	3	12	" 160–165°	
" K	4 and 5c	59	" 120–135° and gum	
		Group II ("middle")		
J	4	23	M.p. 176–180°	Androsterone
D	5a	209	" 180–183°	
H	5b	292	" 180–183°	
K	5c	5	" 176–180°	
D	6a	152	" 140–144°	Etiocholanol-3α-one-17
H	6b	80	" 141–143°	
K	6c	222	" 140–146°	
L	7	41	" 140° and gum	
		Group III ("late")		
N	8	8	Gum	
"	9	91	M.p. 188–189°; etiocholanol-3α-dione-11,17 and gum	
"	10	27	Gum	
M	11	30	"	
Total recovery		1426		
Initial amount		1853		
Recovery, %		77		

* Androsterone equivalents.

TABLE VI

Reproducibility of Chromatographic Separation

Eluates	α-Ketonic fraction	
	Aliquot A	Aliquot B
	per cent	*per cent*
Early	19	20
Middle	58	57
Late	23	23

the different crystalline compounds isolated as well as the fractions which gave a positive Zimmermann reaction but from which no crystalline material was obtained.

Reproducibility of Results—The reproducibility of the results of a typical chromatographic separation of two aliquots of the same urine, when standardized procedures are employed routinely, is shown in Table VI. It will be seen that similar values were obtained by chromatographing two equal portions of an extract from a pool of urine of normal males. The differences between the results of the two separations are not significant.

DISCUSSION

Since the urinary steroids are excreted in conjugated form, the only practicable method for obtaining *all* of the free compounds is by means of acid hydrolysis. Quantitative isolation of the conjugated steroids as such is impractical for the purpose of this investigation, since little is known about the nature of the conjugates. Only a few glucuronides (23–26) and ethereal sulfates (27–29) have been isolated from human urine. Certain conjugated as well as free steroids are affected by relatively mild acid hydrolysis, leading to the formation of such transformation products as $\Delta^{3,5}$-androstadienone-17 (1, 30, 31), $\Delta^{2(\text{or }3)}$-androstenone-17 (1, 27, 32–35), $\Delta^{11(?)}$-androstenol-3α-one-17 (30), and chlorodehydroisoandrosterone (30, 36, 37). It is known that the adrenal cortical-like activity which can be demonstrated in ethylene dichloride extracts of unhydrolyzed urine is lost after acid hydrolysis (38); this may be related in part to the fact that the β-hydroxyl group at C_{11} of the adrenal cortical steroids is eliminated by heating with acid, with the production of the corresponding Δ^9 compounds (39, 40). There seems to be little likelihood that a single method of hydrolysis will be found which will cleave all conjugated steroids without any change in the products of hydrolysis. Transformations may occur at the time the conjugates are cleaved, and, hence, are not necessarily the result of the prolonged action of the hydrogen ion on the unconjugated steroids. The failure to prevent these changes by simultaneous extraction during hydrolysis is in agreement with this concept (41). Nevertheless, the chemical structure of the transformation product is of considerable importance in the recognition and elucidation of the structure of the unaltered compound as excreted in the urine, and may contribute to the development of a method for its isolation. The method of acid hydrolysis described was adopted with its limitations fully in mind. Regardless of the inadequacies of this method, the results obtained are comparable, since the same procedures were used throughout the experiments.

The quantitative determination of the non-ketonic alcoholic steroids by titration of the phthalate half esters presented some difficulty when applied to crude urinary extracts, despite the fact that very acceptable results were obtained in model experiments with pure steroids (Table II). The discrepancy may be due in part to the difficulty encountered in removing the last traces of ethanol and water from large amounts of crude oily extracts. In addition, relatively small aliquots are taken routinely, and small errors are greatly magnified when calculated back to the total weight of the initial extract. The procedure is helpful, however, in following the progress of purification of the "alcoholic fraction;" the results of further application of this method will be dealt with in subsequent reports.

The method of cleaving the phthalate half esters by ester exchange is similar to that used by Chibnall (22). The alcohols recovered from the phthalate half esters were amber-colored semicrystalline oils, while the "non-alcoholic non-ketonic" fractions were viscous red oils and comprised 60 to 90 per cent of the material which did not react with Girard's Reagent T. The separation of these fractions into their components will be the subject of further communications.

The α- and β-ketosteroids were not separated into alcoholic and non-alcoholic components by the phthalic anhydride method since it was desired to avoid treatment with alkali. The separation of the non-alcoholic components was easily effected in the course of the chromatographic analysis and so the use of the phthalate method presented little advantage.

Separation of urinary steroids by chromatographic methods has been employed by many other investigators. The choice of a proper adsorbent for chromatographic separation presents considerable difficulty, since the criteria of selectivity, availability, and unvarying adsorptive capacity are seldom encountered in a single product. Two requirements are very important for the investigations herein reported: (1) the compounds must not be changed by the chromatography, and (2) the reproducible results must be obtained on steroid mixtures regardless of the source of the mixtures. It has been shown, for example, that rearrangements and saponification of esters can occur on alumina (37, 42–43). The experience of this laboratory proves that at least one compound,[2] a β-ketosteroid with an α,β-unsaturated carbonyl group, melting at 238–240°, and with an absorption maximum at 237 mμ, which was isolated from the urine of a patient with an ovarian tumor, was unstable on a column of alumina. A small amount of this compound was obtained by elution with ether containing 1 per cent methanol. Another less pure sample of the same substance with a colorimetric ketosteroid value of 190 mg. was rechromatographed on alumina in an attempt to purify it. The oily eluates which were obtained could not be crystallized and had a colorimetric value of only 90 mg. In complex mixtures of unknown compositions, it is very difficult to determine whether substances have been changed during a chromatographic separation, since the available criteria for the recovery of all of the original material are based on colorimetric measurements or determinations of weight. Of the many adsorbents tested as substitutes for alumina, magnesium silicate was found to give the best recovery in terms of both weight and chromogenic value. This product is not as selective as alumina, or as powerful an adsorbent, but it is extremely useful for material which can be eluted from alumina only with highly polar solvents. Compounds (late group, No. III) which can be eluted from alumina only with methyl alcohol-ether mixtures with some loss of material can be eluted from magnesium silicate with ether-benzene mixtures without loss or obvious alteration.

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[2] Compound B6 (6).

Because the recovery of the early (Group I) and middle (Group II) groups of ketosteroids appeared to be satisfactory when alumina is used, and losses were encountered only in those fractions which were eluted after etiocholanol-3α-one-17, the procedure (as illustrated in the section "Fractional chromatographic separation") was devised to take advantage of the adsorptive properties of both alumina and magnesium silicate (Fig. 1). A preliminary separation was accomplished by chromatographing the crude extract on magnesium silicate-Celite mixture (Chromatogram A, Fig. 1). From this adsorbent the steroids of the early and middle groups were eluted with carbon tetrachloride and benzene-carbon tetrachloride (1:4) mixtures. In the great majority of crude fractions a sharp band of red pigment (indigo red) moved slowly down the column with these solvents (44). Examination of the eluates (usually benzene-carbon tetrachloride (1:4) and (1:1)) in which this red pigment was present revealed that the principal steroid constituent was etiocholanol-3α-one-17. Therefore, all the red pigment eluates and those which were subsequently eluted with more polar solvents were considered to contain the late fractions (Group III) together with etiocholanolone. These were combined and rechromatographed on magnesium silicate. This procedure not only separated the early and middle fractions from the more strongly adsorbed late one, but, what is more important, it accomplished the separation of the relatively labile late fractions without submitting them to the more drastic conditions encountered with aluminum oxide. By combining suitable eluates from different chromatograms and rechromatographing on the appropriate adsorbent (Fig. 1), complex mixtures were resolved into their components (Table IV, Chromatograph H). With some structurally similar compounds, such as $\Delta^{3,5}$-androstadienone-17 and $\Delta^{2\ (\text{or}\ 3)}$-androstenone-17, or isoandrosterone and dehydroisoandrosterone, a partial separation is achieved only after repeated chromatography; however, most substances, including diastereoisomers, were separated easily.

In the course of this investigation over 300 extracts of urine have been separated by chromatographic adsorption procedures. With precisely defined conditions of a fixed sequence of solvents, the steroids are invariably eluted in the same order. Never, for example, has pregnanolone been eluted before allopregnanolone, or etiocholanolone before androsterone. This does not imply that impure samples of a particular steroid will always be eluted with a particular solvent mixture, for the elution of any given compound is markedly influenced by the impurities which accompany that substance. Under identical experimental conditions a pure compound is always eluted from the adsorbent by a particular solvent; an impure substance is eluted by a less polar solvent or mixture of solvents. Nevertheless, the steroids are invariably eluted in the same relative order regardless of the presence or absence of impurities. As purification of the steroids progressed, more and more of the material eluted was crystalline or could be crystallized readily. The characterization of these crystalline compounds is the subject of Paper II (6).

An example of the reproducibility of the results of a chromatographic separation is given in Table VI. This illustrates that very satisfactory results can be achieved with chromatography. Even if much larger errors were accumulated during the sequence of separation procedures, they would be of much smaller magnitude than the variations that arise spontaneously in the state of health or disease.

SUMMARY

1. A description is given of the methods devised to determine the qualitative and quantitative variations between the patterns of steroid excretion in human urine in health and in disease, including neoplastic growth.

2. The methods described include the procedures which have been standardized (*a*) for the collection, hydrolysis, and extraction of urine; (*b*) for the separation of the ether-soluble material into acidic, neutral, and phenolic fractions; (*c*) for the separation of the neutral fraction into ketonic, non-ketonic alcoholic, and non-ketonic non-alcoholic fractions; and (*d*) for the separation of the ketonic and of the non-ketonic alcoholic fractions into the digitonin-precipitable and digitonin-non-precipitable components.

3. The method for the systematic chromatographic adsorption analysis of the ketonic fractions has been described.

4. Examples of the results obtained by the application of these methods have been presented.

5. It is concluded that the methods described are satisfactory for the

separation and isolation of the constituents of the urinary steroid excretion patterns in health and in disease.

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STUDIES IN STEROID METABOLISM

II. IDENTIFICATION AND CHARACTERIZATION OF KETOSTEROIDS ISOLATED FROM URINE OF HEALTHY AND DISEASED PERSONS*

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This paper reports the chemical investigation of steroids isolated from the urines of normal and diseased persons by the methods of extraction, hydrolysis, and partition described in Paper I of this series (8). In the course of the study a total of forty-two apparently homogeneous and non-identical substances have been encountered. Twenty-six of these have been fully characterized and identified; the other sixteen are characterized and are probably new ketosteroids. Twelve of the fully identified compounds have been isolated from urine by previous investigators.[1] The present report thus describes thirty[2] fully or partially characterized substances not encountered previously in human urine. Of the total of forty-two ketosteroids to be described, thirty-five are classified as *α-hydroxy compounds* because they are not precipitated by digitonin; the other seven form sparingly soluble digitonides and are therefore designated as *β-hydroxy compounds.* Reference has been made to only a few examples of the clinical conditions in which a particular compound was excreted; subsequent reports will deal specifically with the steroid excretion patterns associated with various clinical states.

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[1] Citations to the literature are given in the experimental sections dealing with these twelve compounds.

[2] Included in the thirty compounds are the acetates (Compounds A6, A8, and A9) of three previously isolated steroids.

Relationship of Sequence of Elution to Chemical Nature of Compounds

The steroids isolated are described in the experimental section in the sequence of their elution from aluminum oxide and magnesium silicate columns. In Table I the α-ketonic steroids (those not precipitated by digitonin) are listed in the order in which they are eluted in the systematic chromatographic procedure. The β-ketonic steroids are listed in Table II. The fact has been emphasized previously (8) that the sequence of compounds obtained in a standardized chromatogram is always the same. Although a number of the compounds listed in Tables I and II were not regularly encountered in the urine of all individuals studied, if present they were invariably eluted in the relative positions indicated in the tables. As an example, allopregnanol-3α-one-20 (Compound A17) and pregnanol-3α-one-20 (Compound A18) (which are absent from the urine of normal male subjects but occur in the urine of pregnant women) always are eluted after androstanedione-3,17 (Compound A12) and etiocholanedione-3,17 (Compound A13) and prior to androsterone (Compound A20) and etiocholanol-3α-one-17 (Compound A24). It would, of course, be erroneous to assume that a compound must be pregnanol-3α-one-20 because it is eluted between androstanedione-3,17 and androsterone, since the possibility exists that a previously unrecognized compound may be eluted in this position. It has been found that in the presence of large amounts of steroids, of unusual mixtures, or of impurities, the sequence of elution of the compounds is the *same* but each compound is eluted by a less polar solvent than is required to elute it when in the pure state. Accordingly, no conclusion as to the nature of the compound in an eluted fraction can be drawn from the particular eluting solvent employed, nor can a compound be identified by its position in the chromatogram (9). Adequate chemical and physical (10) characterization must be made of each substance before its identity can be established. With complex mixtures such as the ketonic fractions from human urine, separation may not be accomplished if only a single chromatogram is employed. By a procedure which will be termed "fractional chromatographic analysis" involving several chromatograms, such separation may be achieved. The ease of separation of closely related compounds varies considerably. Certain diastereoisomers, for example androsterone–etiocholanol-3α-one-17, can be separated completely after two or three chromatograms. This is true also for the pairs androstanedione-3,17–etiocholanedione-3,17 and allopregnanol-3α-one-20–pregnanol-3α-one-20. In general the separation of the saturated from the unsaturated steroid analogues presents great difficulty (11). Even after numerous chromatograms only a partial separation of mixtures of dehydroisoandrosterone (Compound B4) and isoandrosterone (Compound B3) is possible. Similarly, mixtures of $\Delta^{3,5}$-androstadienone-17 (Compound A2) and $\Delta^{2(\text{or } 3)}$-andros-

TABLE I

Urinary α-Ketonic Steroids

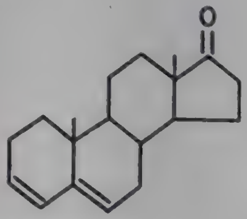
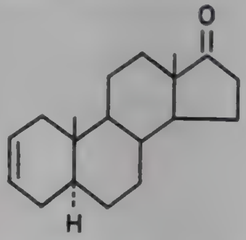
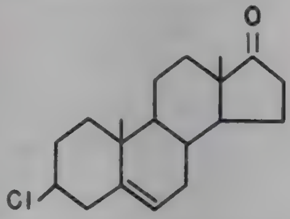
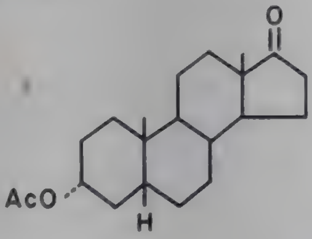
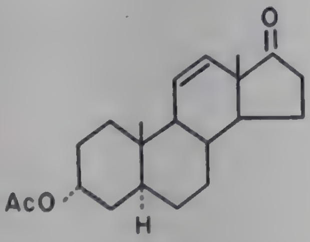
Compound No.	Substance	Empirical formula	Identifying characteristics
A1	248–252°		
A2	 $\Delta^{3,5}$-Androstadienone-17	$C_{19}H_{26}O$	M.p. 78–84°; semicarbazone, m.p. 295–296°; oxime, m.p. 156–161°
A3	 $\Delta^{2\ (or\ 3)}$-Androstenone-17	$C_{19}H_{28}O$	M.p. 109–111°; $[\alpha]_D = +148°$; semicarbazone, m.p. 290–291°; oxime, m.p. 156–158°
A4	 3-Chloro-Δ^5-androstenone-17	$C_{19}H_{27}OCl$	M.p. 155–157°; $[\alpha]_D = +17.6°$
A5	135–136°		
A6	 Etiocholanol-3α-one-17 acetate-3	$C_{21}H_{32}O_3$	M.p. 91–92°; acetate of Compound A24
A7	117–118°	$C_{21}H_{32}O_3$	$[\alpha]_D = +108°$; oxime, $C_{21}H_{33}O_3N$; m.p. 172–177°
A8	 $\Delta^{11(?)}$-Androstenol-3α-one-17 acetate-3	$C_{21}H_{30}O_3$	M.p. 179–180°; $[\alpha]_D = +115°$; free hydroxy ketone, m.p. 176–176.5°; $[\alpha]_D = +122°$

TABLE I—*Continued*

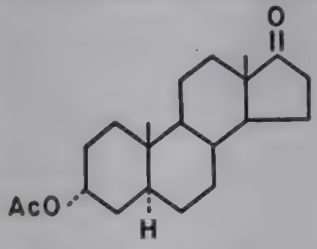
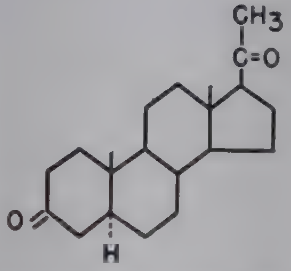
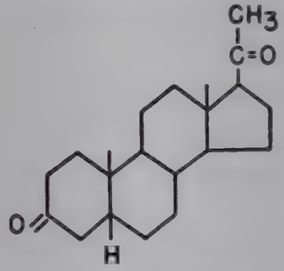
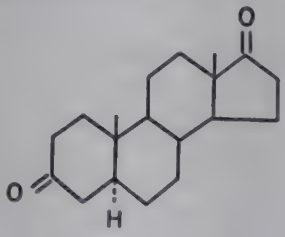
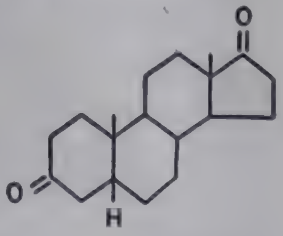
Compound No.	Substance	Empirical formula	Identifying characteristics
A9	 Androsterone acetate	$C_{21}H_{32}O_3$	M.p. 160-162°; $[\alpha]_D = +98.4°$; acetate of Compound A20
A10	 Allopregnanedione-3,20	$C_{21}H_{32}O_2$	M.p. 199-200°; bisdinitrophenylhydrazone, m.p. 207-211°
A11	 Pregnanedione-3,20	$C_{21}H_{32}O_2$	M.p. 120-122°; bisdinitrophenylhydrazone, m.p. 251-253°
A12	 Androstanedione-3,17	$C_{19}H_{28}O_2$	M.p. 132-134°; $[\alpha]_D = +112°$; bisdinitrophenylhydrazone, m.p. 253-256°
A13	 Etiocholanedione-3,17	$C_{19}H_{28}O_2$	M.p. 130-131°; $[\alpha]_D = +112°$; bisdinitrophenylhydrazone, m.p. 263-264°

TABLE I—*Continued*

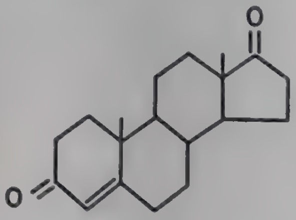
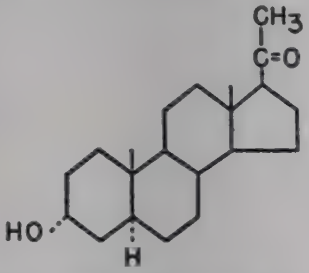
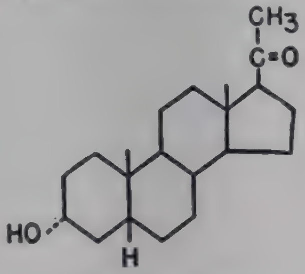
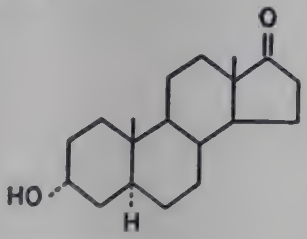
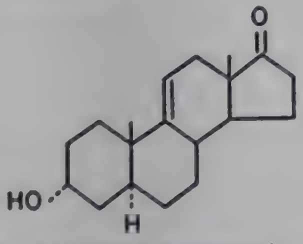
Compound No.	Substance	Empirical formula	Identifying characteristics
A14	 Δ^4-Androstenedione-3,17	$C_{19}H_{26}O_2$	M.p. 170–171.5°; $[\alpha]_D = +196°$; absorption maximum at 239 mμ, log ϵ = 4.20
A15	132–134°		
A16	117–121°		
A17	 Allopregnanol-3α-one-20	$C_{21}H_{34}O_2$	M.p. 174–175°; $[\alpha]_D = +95.7°$; acetate, m.p. 140–142°
A18	 Pregnanol-3α-one-20	$C_{21}H_{34}O_2$	M.p. 148.5–149.5°; $[\alpha]_D = +106°$; acetate, m.p. 94–97°
A19	120–125°		
A20	 Androsterone	$C_{19}H_{30}O_2$	M.p. 184–185°; $[\alpha]_D = +97.7°$; acetate, m.p. 160–162°; benzoate, m.p. 179–180°
A21	 Δ^9-Androstenol-3α-one-17	$C_{19}H_{28}O_2$	M.p. 187–187.5°; $[\alpha]_D = +136°$

TABLE I—*Continued*

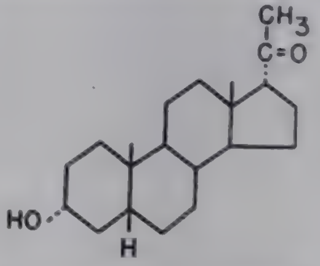
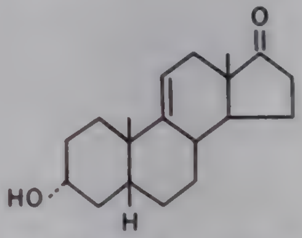
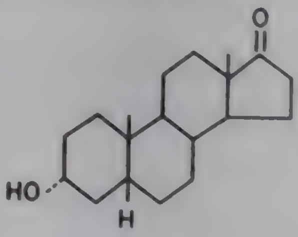
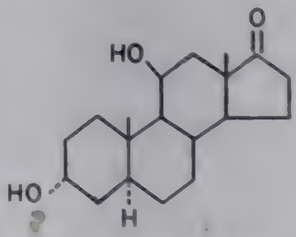
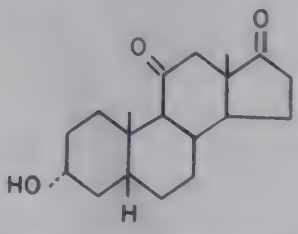
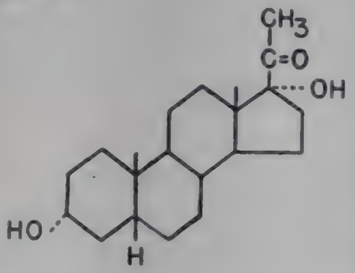
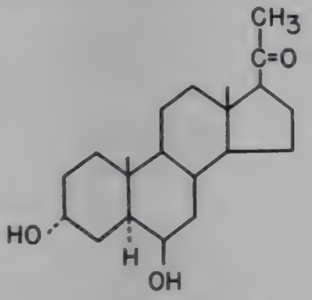
Compound No.	Substance	Empirical formula	Identifying characteristics
A22	 17-Isopregnanol-3α-one-20	$C_{21}H_{34}O_2$	M.p. 143–145°; $[\alpha]_D = -40.9°$; acetate, m.p. 154–157°
A23	 Δ⁹-Etiocholenol-3α-one-17	$C_{19}H_{28}O_2$	M.p. 169–170°; $[\alpha]_D = +151°$
A24	 Etiocholanol-3α-one-17	$C_{19}H_{30}O_2$	M.p. 143–144°, 150°; $[\alpha]_D = +111°$; acetate, m.p. 92–93.5°; benzoate, m.p. 164–164.5°; oxime, m.p. 220–225°; dinitrophenylhydrazone, m.p. 225–226°
A25	185–186°	$C_{21}H_{34}O_3$	
A26	174–175°	$C_{21}H_{32}O_3$	
A27	 Androstanediol-3α, 11β-one-17	$C_{19}H_{30}O_3$	M.p. 199–200°; $[\alpha]_D = +98.4°$; acetate, m.p. 240–242°
A28	 Etiocholanol-3α-dione-11, 17	$C_{19}H_{28}O_3$	M.p. 188–189°; $[\alpha]_D = +95.8°$; acetate, m.p. 163–164°; $[\alpha]_D = +145°$

TABLE I—*Concluded*

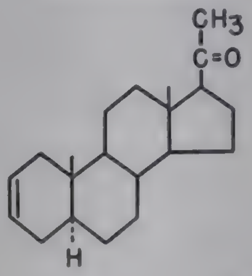
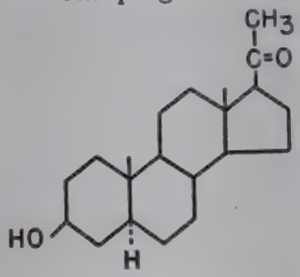
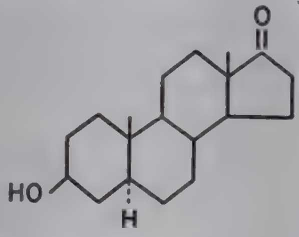
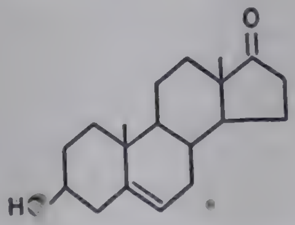
Compound No.	Substance	Empirical formula	Identifying characteristics
A29	235–237°		
A30	 Pregnanediol-3α,17α-one-20	$C_{21}H_{34}O_3$	M.p. 219–219.5°; $[\alpha]_D = +64.5°$; acetate, m.p. 201–202°; oxime, m.p. 247–250°
A31	 Allopregnanediol-3α,6-one-20	$C_{21}H_{34}O_3$	M.p. 195–196°; $[\alpha]_D = +105°$; diacetate, m.p. 155-156°; oxime, m.p. 288–291°
A32	192–194°	$C_{21}H_{34}O_3$	$[\alpha]_D = +66.3°$; diacetate, m.p. 128–129°; dinitrophenylhydrazone, m.p. 234–238°
A33	230–233°		
A34	238–241°		
A35	172–176°		

tenone-17 (Compound A3) are difficult to resolve. Similar experiences were encountered with mixtures of androsterone (Compound A20) and Δ^9-androstenol-3α-one-17 (Compound A21), and with mixtures of Δ^9-etiocholenol-3α-one-17 (Compound A23) and etiocholanol-3α-one-17 (Compound A24). However, the components of these mixtures can be separated by indirect methods such as chromatography of the epoxy derivatives (11).

A correlation can be made between chemical constitution and relative order of elution in this as in other series (12), and Table III presents the generalizations that can be made on the basis of the present experimental results. Derivatives of the allo series (Ring A-B trans) are always eluted before the corresponding derivatives of the normal series (Ring A-B cis). This holds true for the diketones (Compounds A12 and A13), for the C_{21}-monohydroxy ketones (Compounds A17 and A18) and for the C_{19}-monohydroxy ketones (Compounds A20 and A24). Similar rules apply to the small number of ketosteroids which are precipitated by digitonin.

TABLE II

Urinary β-Ketonic Steroids

Compound No.	Substance	Empirical formula	Identifying characteristics
B1	 $\Delta^{2\,(\mathrm{or}\ 3)}$-Allopregnenone-20	$C_{21}H_{32}O$	M.p. 127–129°; $[\alpha]_D = +133°$; an insoluble digitonide; semicarbazone, m.p. 260°
B2	 Allopregnanol-3β-one-20	$C_{21}H_{34}O_2$	M.p. 194–195°; $[\alpha]_D = +91.2°$; acetate, m.p. 144–146°
B3	 Isoandrosterone	$C_{19}H_{30}O_2$	M.p. 175–176°; $[\alpha]_D = +82.0°$; benzoate, m.p. 210–211°
B4	 Dehydroisoandrosterone	$C_{19}H_{28}O_2$	M.p. 137–138°; $[\alpha]_D = +10.4°$; acetate, m.p. 166–166.5°; benzoate, m.p. 256–257°
B5	272–275°		
B6	238–240°	$C_{21}H_{30}O_3$	Absorption maximum at 237.3 mμ, log ε = 4.07; acetate, m.p. 175–176°
B7	209–210°	$C_{21}H_{34}O_3$	$[\alpha]_D = +98.9°$; diacetate, m.p. 102.5–103.5°; oxime, m.p. 275° (decomposition)

The relationship between structure and ease of elution sometimes provides a clue to the nature of an unknown compound. Thus tentative predictions can be made of the structures of those substances listed in Tables I and II that have not as yet been fully characterized. Another useful application is in the search for substances that on biochemical grounds might reasonably be assumed to be present. For instance, the isolation and identification of Δ^9-androstenol-3α-one-17 (Compound A21) and of Δ^9-etiocholenol-3α-one-17 (Compound A23) was greatly facilitated by these predictions.

TABLE III

Order of Elution of Steroid Types

Steroid type	Compound No.
Aluminum oxide column	
1. Monoketones	A2, A3, A4
2. " with one acetoxy group	A6, A7, A8, A9
3. Diketones	
(*a*) Allopregnane series	A10
(*b*) Pregnane "	A11
(*c*) Androstane "	A12
(*d*) Etiocholane "	A13
4. Monohydroxy monoketones	
(*a*) Allopregnane series	A17
(*b*) Pregnane "	A18
(*c*) Androstane "	A20, A21
(*d*) Etiocholane "	A23, A24
Magnesium silicate column	
5. Dihydroxy monoketones, C-19	A27
6. Monohydroxy diketones, C-19	A28
7. Dihydroxy monoketones, C-21	A30, A31*

* The exact order of elution of some of the compounds on magnesium silicate columns has not been finally established in all instances. For example, whereas Compounds A27, A28, and A30 were eluted in this sequence from the same chromatogram, Compound A31 has not been found in the presence of these three compounds and hence its order of elution in relation to them cannot be stated exactly.

Transformation Products—Certain steroids isolated from urines are recognized as transformation products arising in the acid hydrolysis (13, 14), through acetylation in the hydrolysis of the Girard derivative, or from ring enlargement or other rearrangement reaction occurring on the aluminum oxide column (15–17). Molecular rearrangements have not been encountered in the present work, but the other types of changes have been noted.

The unsaturated Compounds A2, A3, A4, A21, A23, and B1 undoubtedly result from the elimination of a hydroxyl group at C_3 or C_{11} during hydrolysis; in each case the double bond introduced can occupy two alternate positions, $\Delta^{2(\text{or } 3)}$ and $\Delta^{9(\text{or } 11)}$. One instance was noted of the acid-catalyzed epimerization at the carbon atom carrying the side chain, 17-isopregnanol-3α-one-20 (Compound A22).

The partial conversion of steroid constituents into their acetates occurs in the course of the hydrolysis of the steroid betaine chloride hydrazones. As a means of minimizing the loss of the more water-soluble steroids, the hydrolysate is extracted continuously with ether, with the result that acetic and hydrochloric acids accumulate in the ethereal solution and produce small amounts of acetylated steroids. Compounds A6 and A9 are acetoxy compounds and Compound A8 has been identified as the acetate of $\Delta^{11(?)}$-androstenol-3α-one-17. In addition to esterification, etherification of steroid alcohols has recently been reported to occur during the treatment with Girard's Reagent T (18).

The recognition of certain compounds as rearrangement or transformation products enables one to deduce the nature of their precursors as excreted in the urine. It must be emphasized, therefore, that a compound isolated from urine loses none of its significance because it is classed as a transformation product.

Structural and Metabolic Relationship of Urinary Steroids to Their Hormonal Precursors

To deduce the source of a given steroid isolated from urine by comparing its structure with that of a known hormone is tempting. There are indications, however, that such inferences may be completely misleading. For example, one cannot assume that urinary steroid metabolites with 19 carbon atoms[3] must arise from the hormone which similarly possesses 19 carbon atoms, testosterone, since androsterone and etiocholanolone also arise from dehydroisoandrosterone (19). One cannot exclude the possibility that some or all of the C-19 compounds are metabolites of C-21 precursors. Furthermore, the C-21 urinary steroids[4] are the excretion products of such different hormones as progesterone, desoxycorticosterone (20–22), and probably other adrenal cortical steroids. The only compounds whose precursors can be determined by chemical analogy with any assurance are those which are "structurally labeled" because they retain a specific structural group characteristic of many adrenal cortical hormones, for example, the C_{11}-OH (Compound A27), the Δ^9 double bond (Compounds A21 and A23), the C_{11} ketone group (Compound A28), or the C_{17} tertiary hydroxyl group (Compound A30).

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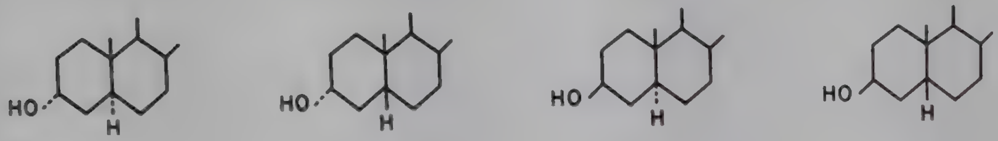
[3] Compounds A2, A3, A4, A6, A7, A8, A9, A12, A13, A14, A20, A21, A23, A24, A27, A28, B3, and B4.

[4] Compounds A10, A11, A17, A18, A22, A25, A26, A30, A31, A32, B1, B2, B6, and B7.

In spite of the difficulty in assigning a precursor to each of the compounds isolated in this investigation, a consideration of the chemical structures of these metabolites reveals certain regularities which imply that the conversions of the steroid precursors to their urinary metabolites follow metabolic rules. For example, three of the four possible isomeric configurations at C_3 and C_5 were found in the C-19 series (androsterone (I), etiocholanol-3α-one-17 (II), and isoandrosterone (III)); three of the four possible isomers were found in the C-21 series (allopregnanol-3α-one-20 (I), pregnanol-3α-one-20 (II), and allopregnanol-3β-one-20 (III)); in *both* the C-19 and C-21 series the same isomeric configuration (Fig. 1, IV) was absent. The two C_5 diastereoisomers of the 3,17-diketones (androstanedione-3,17 and etiocholanedione-3,17) were isolated in the C-19 series; and the two C_5 diastereoisomers of the 3,20-diketones (allopregnanedione-3,20 and pregnanedione-3,20) have been found in the C-21 series. These facts support the thesis that there exist common pathways in steroid metabolism, as has been suggested by several investigators (23, 24).



I II III IV

FIG. 1

Whether similar general principles apply to those C-19 steroids which contain a ketonic group at C_{11} or a C_{11}-OH group remains to be ascertained. It is difficult to assume that the same metabolic rules of conversion apply to the precursors of this C-19 series, because, of the eight possible isomers containing a C_{11}-hydroxyl group, only two (androstanediol-3α,11β-one-17 (Compound A27) and Δ^9-etiocholenol-3α-one-17 (Compound A23)) have been isolated, and of the four possible isomers containing a C_{11}-carbonyl group, only one (etiocholanol-3α-dione-11,17 (Compound A28)) has been found. Whereas two of these C-19 compounds (A27 and A28) are commonly found in the urine of normal individuals, the third (Compound A23) occurs very infrequently.

The isolation of these three C-19 compounds (A23, A27, and A28) structurally labeled at C_{11} strongly suggests that adrenal cortical hormones which possess 21 carbon atoms are degraded to C-19 metabolites, and in general may be precursors of some of the C-19 steroids excreted in urine.

A comparison of the structures of the steroid metabolites with those of known hormones suggests that certain of the possible ketonic intermediates are probably precursors of the metabolites, whereas others are not. Thus, the available data leave no room to suppose that either 3-keto-17-hydroxy C-19 steroids or 3-keto-20-hydroxy C-21 steroids exist as intermediates. On the other hand, several diketones have been encountered: C-19, in the urine of normal persons (androstanedione-3,17, etiocholanedione-3,17); C-21, in the urine of pregnant women (allopregnanedione-3,20) and in the urine of a woman with adrenal cortical hyperplasia (pregnanedione-3,20). There is no conclusive evidence to show whether these diketones are metabolic intermediates or final products, but the fact that biochemical reduction of a C_3 ketonic group always precedes reduction of such a group at C_{17} or C_{20} indicates that the saturated diketones very probably are intermediates in steroid metabolism. Furthermore, since the present evidence indicates that negligible quantities of steroids are excreted in a non-conjugated form, one wonders how diketones of this type which lack hydroxyl groups are conjugated and excreted.

In the case of some of the metabolites listed in Tables I and II, it is difficult to formulate any speculation with regard to the possible precursors. Thus allopregnanediol-3α,6-one-20 (Compound A31) contains a hydroxyl group at C_6, whereas no known steroid hormone possesses an oxygen substituent at this position.

Consideration of the structure of the known hormone precursors leads to certain implications concerning the structure of steroid metabolites isolated from urine. Although several of the steroid hormones contain an α,β-unsaturated carbonyl group in Ring A, urinary metabolites containing this grouping have not been reported prior to the present investigation and have now been isolated in only small amounts. It may be that these substances are present only when the daily steroid excretion is large and some of the precursors are incompletely metabolized, or when the metabolism of the steroids is abnormal. The isolation of two such substances, Δ^4-androstenedione-3,17 (Compound A14) and Compound B6, proves that α,β-unsaturated ketones can be excreted and are therefore present in the circulation. The fact that they are encountered only rarely and in small amounts suggests that in the normal course of metabolism the unsaturated substances are converted to saturated derivatives that are common constituents of urine.

The reduction of a Δ^4-unsaturated diketone can lead to two isomeric saturated diketones, and the isolation of one pair of such substances in the C-19 series (Compounds A12 and A13) and of another pair in the C-21 series (Compounds A10 and A11) indicates that such reduction does indeed represent a metabolic process.

Theoretically, the reduction of both the Δ^4 double bond and the C_3-

carbonyl group can lead to four isomeric configurations, but in both the C-19 and the C-21 series only three of the four possible compounds have been isolated and invariably it is the substance of the configuration IV (Fig. 1) that is missing. The most likely sequence of events leading to the three known types of isomers is saturation of the ethylenic linkage, followed by reduction of the carbonyl group. It is, of course, possible that these isomers are formed from different precursors, and that the resemblance between these urinary metabolites is a superficial one based upon chemical analogy.

One unique structural characteristic of the known adrenal cortical hormones is the presence of a hydroxyl group at C_{21}. Although no urinary steroids containing this distinguishing side chain have been isolated, chemical tests based upon the reducing properties of such ketols have been applied (25–29) and the results have indicated that steroid ketols are present in the urine of both normal and diseased persons. Studies of the metabolic fate of desoxycorticosterone in animals and in man indicate that the organism is capable of reducing the primary alcoholic group at C_{21}, $—COCH_2OH \rightarrow —COCH_3$. A second characteristic grouping present in some of the hormones of the adrenal cortex is the tertiary hydroxyl substituent at C_{17}. The C_{17}-hydroxylated metabolites isolated in this investigation (Compound A30) and in other work (17, 30, 31) were obtained only from the urine of diseased persons but they evidently are derived from hormones of the adrenal cortex. That such steroids have not been found in the urine of normal persons may well be due to the fact that they are intermediates in the normal metabolic degradation of this type of C-21 hormones to C-19 metabolites (32). The presence of a hydroxyl or carbonyl group at C_{11} in three of the compounds isolated from urines (Compounds A23, A27, and A28) is strong evidence that these substances are metabolites of cortical steroid precursors.

Physiological activities characteristic of certain adrenal cortical steroids and apparently associated with the presence of specific chemical groupings (oxygen at C_{11}, ketol side chain, α,β-unsaturated ketone) have been observed in bioassays of various crude fractions extracted from urines (33–35). No metabolites have as yet been characterized which contain the combination of these structural groupings apparently necessary for marked cortical activity (36, 37), although each of these groupings has been found alone in one or more isolated compounds.

An attempt has been made to derive from the chemical structure of the isolated steroid metabolites some knowledge of the nature of their hormonal precursors, and to deduce from special structural features of possible precursors a relationship to the metabolites that have been isolated. Exact knowledge of the relationship between the steroid metabolites and their precursors is fundamental for an evaluation of the rôle of these hormones in normal and abnormal physiology, and for an interpretation of the significance of the urinary steroids in health and in disease.

EXPERIMENTAL[5]

α-Ketosteroids

Hydrocarbon, $C_{29}H_{60}$—Various investigators have described the isolation of hydrocarbons from human urine. Hart and Northrup (38) isolated two substances; one ($C_{27}H_{56}$) was insoluble in methanol and melted at 58–59°, and the other ($C_{25}H_{52}$) was soluble in methanol and melted at 52–54°. Marker (39) described a hydrocarbon ($C_{28}H_{58}$) melting at 63°. A discussion of the chemistry and source of these hydrocarbons has been given recently (40).

During the course of this investigation a waxy substance has been isolated from many different urines. It is usually the first material to be eluted from alumina with ligroin. Soluble in ether, ligroin, or ethyl acetate and rather insoluble in methanol, it was crystallized as waxy plates from acetone, m.p. 53–55°. It was sublimed at 10^{-4} mm. at 70–80° for analysis.

Analysis—$C_{29}H_{60}$.	Calculated.	C 85.18, H 14.81
	Found.	" 84.89, " 15.00
		" 84.75, " 15.25

Compound A1. 248-252°—This substance was isolated in traces from the urine of two patients, one with cancer of the breast (Case CaBF10), and the other with cancer of the prostate (Case CaP1). It was eluted from alumina with ligroin and was crystallized from dilute methanol, m.p. 248-252°.

Compound A2. $\Delta^{3,5}$-Androstadienone-17—This compound was previously isolated from the urine of a man (14) and a woman (41) each having adrenal cortical tumors. In this study it was found in the urines of most normal men and women and of subjects with adrenal dysfunction (1). In most instances this substance was recognized by its absorption maximum at 240 mμ and then isolated as its insoluble semicarbazone; the crystallization of the free ketone was difficult and uncertain. A few mg. of the compound, eluted from alumina with ligroin-benzene mixtures (4:1), were isolated from the urine of a girl with adrenogenital syndrome due to an adrenal hyperplasia (Case AHF2). It was recrystallized from dilute methanol and melted at 78–84°. When mixed with an authentic sample (m.p. 84–87°), it melted at 78–84°.

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[5] The microanalyses reported herein were performed by the courtesy of Dr. A. Elek, The Rockefeller Institute for Medical Research, New York. The melting points were taken in a Hershberg melting point apparatus and are correct to about ±1°.

The semicarbazone was recrystallized from dioxane and melted in a preheated bath at 295–296° with decomposition.

Analysis—$C_{20}H_{29}N_3O$. Calculated, N 12.84; found, N 12.63

When mixed with an authentic sample, it melted at 295°. Dr. R. N. Jones, to whom we are greatly indebted, measured the ultraviolet spectrum of this semicarbazone in dioxane. Since it showed the same absorption maximum as the free ketone, it could be differentiated by this procedure from the semicarbazone of Δ^2-androstenone-17 (Compound A3), which has a similar melting point (m.p. 290–291°).

The oxime prepared from a less highly purified sample melted at 152–157°. It did not depress the melting point of an authentic sample of $\Delta^{3,5}$-androstadienone-17 oxime melting at 156–161°.

Compound A3. $\Delta^{2(or\ 3)}$-Androstenone-17—This substance was previously isolated from the pooled urines of normal persons (42, 43), from the urine of ovariectomized women (44), from the urine of a man with cancer (42), and from the urine of a man with testicular tumor (45). In the investigation herein reported it was found in the urine of normal men and women and in the urine of subjects with adrenal dyscrasias (1) (Cases AHF1 and AHM4). It was eluted from alumina with benzene and was recrystallized from aqueous methanol; it melted at 109–111°. Frequently samples were obtained which melted as high as 115–119°, and did not depress the melting point of the lower melting samples. The possibility exists that these higher melting fractions (reported also by Hirschmann (44)) contain other isomers of androstenone, such as the Δ^3 (since the location of the double bond in the isomer melting at 109–111° is still not certain), or the $\Delta^{2(or\ 3)}$-etiocholenone-17. When mixed with $\Delta^{2(or\ 3)}$-androstenone-17 (m.p. 108–108.5°), kindly supplied by Dr. H. Hirschmann, there was no depression in the melting point. $[\alpha]_D^{28} = +148°$ (11.50 mg. in 2.98 ml. of 95 per cent ethanol).

Analysis—$C_{19}H_{28}O$. Calculated, C 83.77, H 10.36; found, C 83.93, H 10.37

Hirschmann (44) reported a melting point of 107–109°; $[\alpha]_D^{28} = +152°$.

The oxime was recrystallized from dilute methanol and melted at 156–158°; Pearlman (42) reported 153–154°.

Analysis—$C_{19}H_{29}ON$. Calculated, C 79.40, H 10.16; found, C 79.47, H 10.03

The melting point of the oxime was depressed on admixture with the oxime of $\Delta^{3,5}$-androstadienone-17. The semicarbazone of $\Delta^{2(or\ 3)}$-androstenone-17 decomposed at 290–291.5° and showed no absorption in the ultraviolet region of the spectrum.

Compound A4. 3-Chloro-Δ^5-androstenone-17—This substance has been previously isolated from pooled normal male urine (46) and from the urine of patients with an adrenal cortical tumor (31, 41). In this investigation 36 mg. of the compound were isolated from the urine of a man with feminism due to an adrenal hyperplasia (Case AHM1). It was eluted from alumina with ligroin-benzene (1:4) mixture and when crystallized from dilute methanol it formed needles melting at 155–157°. A mixture of this compound with an authentic sample of chlorodehydroisoandrosterone showed no depression of the melting point. $[\alpha]_D^{25} = +17.6° \pm 6°$ (5.09 mg. in 2.98 ml. of chloroform). Butenandt and Grosse (46) reported a melting point of 155–157°; $[\alpha]_D^{21} = +14°$ (chloroform).

Analysis—$C_{19}H_{27}OCl$.	Calculated.	C 74.36, H 8.89
	Found.	" 73.77, " 8.77
		" 74.99, " 8.92

Compound A5. 135–136°—About 3 mg. of this substance were isolated from a pool of human pregnancy urine (Case PP1). It was eluted from alumina with ligroin-benzene (3:1) mixture. On recrystallization from dilute methanol, plates were obtained which melted at 135–136°, and which depressed the melting point of Compound A15, m.p. 132–134°.

Compound A6. Etiocholanol-3α-one-17 Acetate-3—This compound was isolated from the pooled urine of normal men (Case NMP1). It was eluted from alumina with benzene-ligroin (1:3) and after two recrystallizations from ligroin (b.p. 30°) melted at 91–92°. It did not depress the melting point of an authentic sample of etiocholanolone acetate. The semicarbazone of the urinary substance melted at 254–255.5° and likewise did not depress the melting point of the semicarbazone made from etiocholanolone acetate.

Hydrolysis of the isolated steroid with potassium carbonate in aqueous methanol solution yielded etiocholanol-3α-one-17, m.p. 143–144.5°; $[\alpha]_D^{23} = +115° \pm 5°$ (3.98 mg. in 2.00 ml. of ethanol).

Compound A7. 117–118°—This compound was isolated from the urine of several normal persons. It was eluted from alumina with ligroin-benzene (1:1) together with $\Delta^{11(?)}$-androstenol-3α-one-17 acetate-3 (Compound A8). The mixture of these two compounds was dissolved in ether, and ligroin was added to the solution until incipient turbidity. The substance which crystallized first was Compound A8, which is described below. The mother liquor was concentrated to dryness and the residue was recrystallized several times from ligroin. A product melting at 117–118° was obtained. Approximately 30 mg. of the compound were isolated from a 154 day urine collection of a normal subject. When mixed with $\Delta^{2(\text{or } 3)}$-androstenone-17, m.p. 110°, the melting point was depressed. The test for unsaturation with tetranitromethane was negative. $[\alpha]_D^{30} = +108° \pm 5°$ (6.34 mg. in 2.98 ml. of ethanol).

The infra-red spectrum of this substance indicates that it, like Compounds A6, A8, and A9, also contains an acetoxy group.

Analysis—$C_{21}H_{32}O_3$. Calculated, C 75.85, H 9.71; found, C 75.88, H 9.86

The oxime prepared in the usual way was crystallized from dilute methanol as tiny needles. It melted at 172–177°.

Analysis—$C_{21}H_{33}O_3N$. Calculated, C 72.58, H 9.57; found, C 72.34, H 9.57

Compound A8. $\Delta^{11(?)}$-Androstenol-3α-one-17 Acetate-3—Found in the urine of several normal persons and in the urine of a male eunuch with an adrenal hyperplasia (Case AHM4), this compound was isolated together with Compound A7, m.p. 117–118°. Both compounds were eluted from alumina with ligroin-benzene (1:1) and their separation is described above. Compound A8 crystallized as long needles from ligroin or acetone-water mixtures and melted at 179–180°; $[\alpha]_D^{27} = +115° \pm 2°$ (9.45 mg. in 2.00 ml. of ethanol).

Analysis—$C_{21}H_{30}O_3$. Calculated. C 76.32, H 9.15, acetyl 13.02
Found. " 75.65, " 9.52, " 13.06

It gave a yellow color with tetranitromethane and in the modified Zimmermann test 1.0 mg. gave a color equivalent to 1.1 mg. of dehydroisoandrosterone. When mixed with the acetate of $\Delta^{11(?)}$-androstenol-3α-one-17 (m.p. 177–179°; $[\alpha]_D^{24} = +114° \pm 5°$ (in ethanol)) (41), the mixture melted at 175–181°.

40 mg. of $\Delta^{11(?)}$-androstenol-3α-one-17 acetate-3 were dissolved in 2.5 ml. of methanol and 20 mg. of potassium carbonate dissolved in 0.3 ml. of water were added to the solution. After standing overnight the mixture was heated under a reflux for 1 hour and then poured onto ice and water. The precipitate was filtered and washed and, after drying, weighed 29 mg. After two recrystallizations from acetone-ligroin, the compound melted at 176–176.5°; $[\alpha]_D^{28} = +122° \pm 3°$ (6.88 mg. in 2.00 ml. of ethanol). When admixed with $\Delta^{11(?)}$-androstenol-3α-one-17 (m.p. 180–182°; $[\alpha]_D^{28} = +122° \pm 2°$) (41), it melted at 176–177°.

The material used for rotation (6.88 mg.) was dissolved in 0.5 ml. of pyridine and 0.05 ml. of benzoyl chloride was added. The solution was heated for 1 hour on the steam bath, after which it was poured onto ice and water. The oil which precipitated was extracted with ethyl acetate. The extract was washed with dilute sulfuric acid solution, with 10 per cent sodium carbonate solution, and with water. After drying over sodium sulfate, the solvent was removed. A yellow oil was obtained which was purified by chromatographing on alumina. The fraction (6 mg.) eluted with benzene was recrystallized twice with ligroin, and melted at 163–164°. There was no depression when the product was mixed with $\Delta^{11(?)}$-androstenol-3α-one-17 benzoate-3 prepared by Wolfe, Fieser, and Friedgood (41).

Compound A9. Androsterone Acetate—This compound was isolated from the pooled urine of normal men (Case NMP1). It was eluted from alumina with ligroin-benzene (1:1) and when recrystallized from ether-ligroin melted at 161–166°. On hydrolysis with potassium carbonate in aqueous methanol solution, androsterone, m.p. 183–185°, was obtained. $[\alpha]_D^{16.5} = +98.4° \pm 1°$ (10.1 mg. in 1.01 ml. of ethanol). There was no depression in melting point when mixed with an authentic sample of androsterone.

Compound A10. Allopregnanedione-3,20—This compound has not been isolated previously from human urine. It was obtained from a pool of human pregnancy urine (Case PP1) and was eluted from alumina with benzene. When crystallized from an acetone-water (1:2) mixture, it melted at 199–200° and upon admixture with an authentic sample of allopregnanedione-3,20, it gave no depression in melting point.

The orange bis-2,4-dinitrophenylhydrazone of this compound was prepared and recrystallized from absolute methanol. It melted at 207–211° and did not depress the melting point (208–211°) of the bisdinitrophenylhydrazone prepared from an authentic sample of allopregnanedione-3,20.

Compound A11. Pregnanedione-3,20—About 3 mg. of this substance were isolated from the urine of a woman with adrenal hyperplasia of the adrenogenital type (Case AHF2). It was eluted from alumina with benzene and when recrystallized from ligroin (b.p. 30°) melted at 120–122°. When mixed with an authentic sample of pregnanedione, m.p. 120–121°, there was no depression in melting point. The infra-red spectra of both specimens were identical.

The bisdinitrophenylhydrazone of the pregnanedione isolated from urine melted at 251–253° and did not depress the melting point of the hydrazone, m.p. 250–252°, made from authentic pregnanedione.

Compound A12. Androstanedione-3,17—This substance has not been previously reported to be present in human urine. About 50 mg. were isolated from a pool of urine from normal men (Case NMP1) and women (Case NFP1) and from the urine of a normal man (Case NM4). The compound was eluted from alumina with ligroin-benzene (1:3) and after recrystallization from either ether-ligroin or aqueous acetone, it crystallized as silky needles melting at 132–134°; $[\alpha]_D^{31} = +112.5° \pm 2.5°$ (4.08 mg. in 2.00 ml. of ethanol). It did not depress the melting point of an authentic sample of androstanedione-3,17. Butenandt *et al.* (47) reported a melting point of 133°; $[\alpha]_D^{20} = +111°$ (in ethanol). The sample for analysis was prepared by sublimation in high vacuum at 100°.

Analysis—$C_{19}H_{28}O_2$. Calculated, C 79.12, H 9.78; found, C 79.24, H 9.87

The bisdinitrophenylhydrazone was prepared from 6 mg. of the dione and 10 mg. of 2,4-dinitrophenylhydrazine dissolved in 3 ml. of methanol containing 2 drops of concentrated hydrochloric acid by heating under a reflux for 1 hour. The orange precipitate was filtered and recrystallized from chloroform-methanol mixture, m.p. 253–256°. It did not depress the melting point of the bisdinitrophenylhydrazone prepared from an authentic sample of androstanedione-3,17.

Analysis—$C_{31}H_{36}O_8N_8$. Calculated, N 17.28; found, N 17.00

Compound A13. Etiocholanedione-3,17—This substance was found in traces in the urine of normal men and women, in the urines of three women with hypertension (Cases HF3, HF4, and HF5), and of one woman with Cushing's disease (Case CSF2). The crystalline diketone was isolated from the fractions eluted from alumina with benzene. On recrystallization from ligroin it melted at 130–131°; $[\alpha]_D^{26} = +112° \pm 7°$ (2.77 mg. in 2.00 ml. of ethanol). It did not depress the melting point of an authentic sample. The analytical sample was prepared by sublimation in high vacuum.

Analysis—$C_{19}H_{28}O_2$. Calculated, C 79.12, H 9.78; found, C 79.13, H 9.88

Butenandt *et al.* (48) reported a melting point of 128°; $[\alpha]_D^{21} = +115.2°$ (in ethanol).

The bisdinitrophenylhydrazone melted at 263–264° and did not depress the melting point of an authentic sample (4).

Analysis—$C_{31}H_{36}O_8N_8$. Calculated, N 17.28; found, N 17.00

Compound A14. Δ^4-Androstenedione-3,17—This compound has not been reported to be present in human urine. It was isolated in this study from the urine of a man with adrenal hyperplasia (Case AHM1). The compound was eluted from alumina with benzene and crystallized from ligroin (b.p. 60°). After several recrystallizations it melted at 170–171.5°; $[\alpha]_D^{23} = +196° \pm 4°$ (2.55 mg. in 2.00 ml. of ethanol). When mixed with an authentic sample of Δ^4-androstenedione-3,17, the mixture melted at 170–172°. The compound showed an absorption maximum at 239 mμ; $\log \epsilon = 4.20$ (in ethanol). Its infra-red absorption spectrum was identical with that of the authentic sample (10).

Compound A15. 132–134°—About 4 mg. of this substance were isolated from the urine of a male eunuch with adrenal hyperplasia (Case AHM4). This compound was eluted from alumina with benzene and depressed the melting point of Compound A5, m.p. 135–136°.

Compound A16. 117–121°—About 6 mg. of this substance were isolated from the extract of a large pool of human pregnancy urine (Case PP1). It was eluted from alumina with ether-benzene (1:3) mixture and when recrystallized from ligroin melted at 117–121°. It depressed the melting point of Compound A7, m.p. 117–118°, of Compound A19, m.p. 120–125°, and of pregnanedione-3,20, Compound A11, m.p. 120–122°.

Compound A17. Allopregnanol-3α-one-20—This compound has been previously isolated from human pregnancy urine (49) and in this investigation it was likewise isolated from pregnancy urine (Case PP1). It was eluted from alumina with ether-benzene (1:9) mixture immediately preceding its isomer, pregnanolone (Compound A18). It was recrystallized from aqueous methanol to a melting point of 174–175°; $[\alpha]_D^{27} = +95.7° \pm 2°$ (15.57 mg. in 2.98 ml. of ethanol), also $[\alpha]_D^{25} = 96.6° \pm 1°$ (22.51 mg. in 2.98 ml. of ethanol). Marker *et al.* (49) reported a melting point of 162–164°; $[\alpha]_D^{30} = +91.0°$ (in ethanol). Butenandt and Heusner (50) reported 173–174°.

Analysis—$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.34, H 10.89

The acetate was prepared and when recrystallized from ligroin melted at 140–142°. Oxidation of the hydroxy ketone with chromium trioxide gave allopregnanedione-3,20, which, after several recrystallizations from acetone, melted at 201–203°; $[\alpha]_D^{24} = +117° \pm 4°$ (7.65 mg. in 2.98 ml. of ethanol). It did not depress the melting point of an authentic sample. Butenandt and Mamoli (51) reported a melting point of 200.5°; $[\alpha]_D^{20} = +126.9°$ (in ethanol).

Compound A18. Pregnanol-3α-one-20—This compound has been isolated previously from the urine of pregnant women (52, 53). In this study it was isolated from the urine of pregnant women (Case PP1), of a male eunuch with adrenal hyperplasia (Case AHM4), and of two women with adrenogenital syndrome due to adrenal hyperplasia (Cases AHF1 and AHF2). Each subject excreted about 40 mg. in a 24 day collection of urine. The compound was isolated in large amounts from a pool of human pregnancy urine. It was eluted from alumina with ether-benzene (1:9) mixture immediately following allopregnanol-3α-one-20 (Compound A17). When crystallized from benzene, it formed long needles melting at 148.5–149.5°; $[\alpha]_D^{26} = +107° \pm 2°$ (20.14 mg. in 2.98 ml. of ethanol). Butenandt and Müller (54) reported a melting point of 148–149°; $[\alpha]_D^{19} = +113.8° \pm 1.2°$ (in ethanol). When mixed with a sample of pregnanol-3α-one-20 kindly supplied by Dr. W. H. Pearlman, there was no depression of the melting point.

Analysis—$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.09, H 10.52

The acetate was recrystallized from aqueous methanol and melted at 94–97°. A mixture with an authentic specimen melted at 95–98°.

Compound A19. 120–125°—About 15 mg. of this substance were isolated from the extract of a large pool of human pregnancy urine (Case PP1). It was eluted from alumina with ether-benzene (1:9) mixture together with androsterone, from which it was separated by fractional crystallization. Recrystallization from ligroin yielded a sample melting at 120–125°, which depressed the melting point of pregnanedione-3,20 and of Compound A16, m.p. 117–121°.

Compound A20. Androsterone—Androsterone has been previously isolated from the urine of normal men and women (13, 43, 55–58), castrated men (59), ovariectomized women (44), pregnant women (52), women with breast cancer (60), and of patients with testicular tumor (45), adrenocortical tumor (41, 61), and adrenocortical hyperplasia (31). In this investigation it was isolated from every urine with the exception of certain patients with cancer, with Cushing's syndrome, and of a patient with an adrenal tumor. This substance is the first constituent of the "middle" (8) fraction, and is usually eluted from alumina with ether-benzene (1:9) mixtures. For analysis it was crystallized from methanol-water, ligroin-acetone, and finally from acetone. It melted at 184–185°, $[\alpha]_D^{27} = +97.7° \pm 2°$ (14.24 mg. in 2.98 ml. of ethanol); Ruzicka *et al.* (62) reported a melting point of 182–183°, $[\alpha]_D = +94.6°$ (ethanol); Butenandt (63) 178°, $[\alpha]_D^{20} = +93°$ (ethanol); and David and Freud (64) 180–181.5°, $[\alpha]_D = +96°$ (ethanol). It gave no depression in melting point when mixed with an authentic sample of androsterone.

Analysis—$C_{19}H_{30}O_2$. Calculated, C 78.57, H 10.40; found, C 78.97, H 10.04

The acetate was recrystallized from aqueous methanol and from ligroin and melted at 160–162°; $[\alpha]_D^{28} = +87.4° \pm 3°$ (8.24 mg. in 2.00 ml. of ethanol). It did not depress the melting point of an authentic sample. Androsterone benzoate, recrystallized from methanol, melted at 179–180°; $[\alpha]_D^{28} = +3.4° \pm 5°$ (5.83 mg. in 2.00 ml. of ethanol).

Compound A21. Δ^9-Androstenol-3α-one-17—This substance, which has been found previously in human urine (65), was isolated in this investigation (7) from the urine of normal men (Case NMP2). It was eluted from alumina with ether-benzene (1:4) mixture and crystallized from ligroin (b.p. 60°) as long needles melting at 187–187.5°; $[\alpha]_D^{23} = +136° \pm 5°$ (4.11 mg. in 2.00 ml. of ethanol). From the corresponding 11-β-hydroxy (Compound A27), Mason and Kepler (31) synthesized Δ^9-androstenol-3α-one-17 with a melting point of 189–190° and a specific rotation of $+140° \pm 2°$ (in ethanol). The melting point of the steroid isolated in this laboratory from urine was not depressed on admixture with the authentic compound generously made available to us by Dr. H. L. Mason. The infra-red spectra of the two samples were identical.

Compound A22. 17-Isopregnanol-3α-one-20[6]—This compound, prepared by Moffett and Hoehn (66), has not been hitherto isolated from natural sources. It was found in a pool of human pregnancy urine (Case PP1) and was eluted from alumina with ether-benzene (1:10). When recrystallized from ether-ligroin, it melted at 143–145°; $[\alpha]_D^{28} = -38.9°$ (9.97 mg. in 2.98 ml. of ethanol). Moffett and Hoehn (66) reported 142.5–144°; $[\alpha]_D^{32} = -40.9° \pm 5°$.

Analysis—$C_{21}H_{34}O_2$. Calculated, C 79.18, H 10.77; found, C 79.39, H 10.81

The compound depressed the melting point of both etiocholanol-3α-one-17, m.p. 143-144°, 150°, and pregnanol-3α-one-20, m.p. 148–149°. There was no depression when it was mixed with 17-isopregnanol-3α-one-20. The acetate when recrystallized from dilute methanol melted at 154–157°. When mixed with the acetate of 17-isopregnanol-3α-one-20, there was no depression in melting point.

Compound A23. Δ⁹-Etiocholenol-3α-one-17[7]—This substance was isolated from the urine of a patient with cancer of the breast (Case CaBF16). Its presence has been established also in the urine of certain patients with cancer of the prostate, lymphatic leucemia, essential hypertension, and Cushing's syndrome. It was eluted from alumina with ether-benzene (3:7) mixture and after several recrystallizations from acetone melted at 169–170°; $[\alpha]_D^{22} = +151° \pm 4.5°$ (2.25 mg. in 2.00 ml. of acetone). It was identified by direct comparison with an authentic sample prepared by Dr. L. H. Sarett, who kindly made it available to us.

Compound A24. Etiocholanol-3α-one-17—This substance was first isolated by Butler and Marrian (30) from the urine of a woman with virilism. It has also been isolated from the urine of normal men (43, 55) and normal women (57); from the urine of castrated men (59) and castrated women (44, 61); from the urine of women with adrenal tumors (31, 41) and with adrenal cortical tumor and adrenal cortical hyperplasia (31, 30); as well as from the urine of women with breast cancer (60). This compound was found in most of the urine specimens examined. It was always eluted from alumina after its isomer androsterone with ether-benzene (1:4) mixture. It was recrystallized from benzene-ligroin mixtures to a constant melting point of 143–144° with a second melting point of 150°; $[\alpha]_D^{24} = +111° \pm 3°$ (10.48 mg. in 2.00 ml. of ethanol). When mixed with an authentic sample, its melting point was not depressed..

Analysis—$C_{19}H_{30}O_2$. Calculated, C 78.57, H 10.40; found, C 78.74, H 10.27

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[6] We are indebted to Dr. W. H. Hoehn for having suggested that Compound A22 was identical with his synthetic product and for making available to us this compound and its acetate.

[7] A preliminary report of this substance has been made (6).

The acetate after several recrystallizations from aqueous methanol melted at 92–93.5° and there was no depression of the melting point when this preparation was mixed with an authentic sample of etiocholanol-3α-one-17 acetate.

The benzoate prepared in the usual manner was recrystallized from methanol and melted at 164–164.5°; $[\alpha]_D^{28} = +111° \pm 3°$ (8.11 mg. in 2.00 ml. of ethanol). The oxime of etiocholanol-3α-one-17 was recrystallized from ethyl acetate and melted at 220–225°; $[\alpha]_D^{22} = +66.2° \pm 7°$ (2.72 mg. in 2.00 ml. of ethanol). The dinitrophenylhydrazone melted at 225–226°.

Compound A25. 185–186°—This compound was isolated from the urine of a girl with adrenogenital syndrome due to adrenal hyperplasia (Case AHF2). It was eluted from alumina with acetone-ether (1:9) mixture. When recrystallized from benzene-ligroin (1:5) mixture, it formed clusters of silky needles melting at 185–186°. It depressed the melting point of Compound A28, m.p. 188–189°, and also of $\Delta^{11(?)}$-androstenol-3α-one-17, m.p. 180°.

Analysis—$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10.25; found, C 75.41, H 10.69

Compound A26. 174–175°—From the urine of a girl with adrenogenital syndrome due to adrenal hyperplasia (Case AHF13) about 10 mg. of a compound melting at 174–175° were obtained. It was eluted from alumina with ether-benzene (1:1) mixture and was crystallized from a mixture of ether-ligroin. This substance depressed the melting point of Compound A8, m.p. 179–180°, and of Compound A35, m.p. 172–176°.

Analysis—$C_{21}H_{32}O_3$. Calculated, C 75.85, H 9.71; found, C 76.03, H 9.77

Compound A27. Androstanediol-3α,11β-one-17[8]—This substance has been isolated previously by Mason and Kepler from the urine of normal women (31), of three patients with adrenal cortical tumors, and of four patients with adrenal cortical hyperplasia (31, 68). It has also been isolated from a young woman suffering from virilism (65). In this investigation it was isolated from the urine of a male eunuch with adrenal hyperplasia (Case AHM4) and also from the urine of patients with Cushing's syndrome (Case CSFP1). It was eluted from alumina with acetone-ether (1:4) mixtures and from magnesium silicate-Celite with ether-benzene (3:7) mixtures. The substance crystallized from either acetone-ligroin or benzene-ligroin mixtures as small needles melting at 199–200°; $[\alpha]_D^{22} = +98.4° \pm 3°$ (3.66 mg. in 2.00 ml. of ethanol).

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[8] Mason and Kepler (31) first drew attention to the fact that the melting point and empirical formula of this compound, as published in a previous report from this laboratory (2), were identical with that of the androstanediol-3α,11β-one-17 they isolated from human urine.

Analysis—$C_{19}H_{30}O_3$. Calculated, C 74.44, H 9.87; found, C 74.30, H 10.21

This substance, when mixed with a specimen of androstanediol-3α,-11β-one-17, kindly supplied by Dr. H. L. Mason, showed no depression in the melting point. The infra-red spectra of both samples were identical. The acetate, prepared with acetic anhydride in pyridine solution, was recrystallized from acetone-ligroin and melted at 240–242°. When mixed with an authentic sample of the acetate kindly made available by Dr. R. I. Dorfman, there was no depression in melting point. The infra-red spectra of the two specimens were identical.

Compound A28. Etiocholanol-3α-dione-11,17 (5)—This compound was isolated from the majority of urines examined. It was eluted from alumina with ether containing 0.5 per cent methanol and from magnesium silicate with ether-benzene (1:3) mixtures. It was recrystallized from ether-ligroin mixtures to give long needles, m.p. 188–189°, $[\alpha]_D^{27} = +95.8° \pm 3°$ (9.64 mg. in 2.98 ml. of ethanol), which did not depress the melting point of a synthetic sample of etiocholanol-3α-dione-11,17, generously made available to us by Dr. L. H. Sarett.

Analysis—$C_{19}H_{28}O_3$.	Calculated.	C 74.97, H 9.27
	Found.	" 75.00, " 9.54
		" 75.28, " 9.29

The acetate was prepared in the usual way from acetic anhydride in pyridine and was purified by chromatographing on alumina. The fractions which were eluted with benzene-ligroin (1:1) mixtures were recrystallized from ether-ligroin and melted at 163–164°; $[\alpha]_D^{18} = +145.4° \pm 2°$ (5.86 mg. in 1.04 ml. of ethanol).[9] The acetate did not depress the melting point of the synthetic etiocholanol-3α-dione-11,17 acetate-3, m.p. 163–164°.

Analysis—$C_{21}H_{30}O_4$. Calculated, C 72.81, H 8.72; found, C 72.65, H 8.71

This monoacetate was recovered unchanged after treatment with an excess of chromic acid in acetic acid at room temperature for 5 hours. However, under the same conditions the free diketo alcohol was oxidized to the triketone etiocholanetrione-3,11,17. The oily oxidation product was chromatographed on alumina and the fractions eluted by benzene-ligroin (1:1) were recrystallized from ether-ligroin and melted at 132–133°; $[\alpha]_D^{18} = +148.5° \pm 1°$ (10.29 mg. in 1.01 ml. of ethanol).

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[9] The rotation of etiocholanol-3α-dione-11,17 acetate-3 was given (69) as $[\alpha]_D^{20} = +176°$ in acetone; however, the rotation of the sample prepared in this laboratory in acetone was $[\alpha]_D^{25} = +155° \pm 2.5°$ (4.12 mg. in 2.00 ml. of acetone). Dr. Sarett kindly made available a sample of his acetate and the rotation was repeated. It was $[\alpha]_D^{23} = +157° \pm 1.5°$ (6.43 mg. in 2.00 ml. of acetone).

Analysis—$C_{19}H_{26}O_3$. Calculated, C 75.46, H 8.67; found, C 75.38, H 8.74

Compound A29. 235–237°—A small amount of this substance was obtained from a pool of human pregnancy urine (Case PP1) and also from the urine of a woman with cancer of the breast (Case CaBF10). It was eluted from alumina with 0.5 per cent methanol in ether or acetone-ether mixture (1:4). A mixture with Compound A33, m.p. 230–233°, melted at 185–220°. When mixed with Compound A34, m.p. 238–241°, the mixture melted at 206–214°.

Compound A30. Pregnanediol-3α,17α-one-20—This compound, first isolated in this laboratory (4), was obtained from the urine of a woman with adrenogenital syndrome due to adrenal cortical hyperplasia (Case AHF2), of a cryptorchid male (Case EM1), and of a woman with adrenal cortical tumor (Case ATF2). The method of isolation, the chemical characterization, and the elucidation of the structure of this compound have already been described (4).[10] In this compound the ketonic group at C_{20} is reactive and forms an oxime, whereas the tertiary hydroxyl group at C_{17} is unreactive and cannot be acetylated. These chemical properties are similar to those exhibited by the 17-hydroxy-20-ketosteroids isolated from adrenal glands. Because von Euw and Reichstein (70) have shown that the C_{17}-OH group of these adrenal substances is in the α configuration, the tertiary hydroxyl group of the urinary steroid must also be in the same steric arrangement.

Compound A31. Allopregnanediol-3α,6-one-20, M.P. 195–196°, and Its Conversion to Allopregnanetrione-3,6,20—About 300 mg. of this compound were isolated from the extract of about 1000 gallons of pooled urine from pregnant women (Case PP1). It was eluted from alumina with 2 per cent methanol in ether. It crystallized as needles from benzene or acetone and melted at 195–196°; $[\alpha]_D^{26} = +105° \pm 1°$ (51.15 mg. in 2.98 ml. of ethanol).

Analysis—$C_{21}H_{34}O_3$.	Calculated.	C 75.40, H 10.25
	Found.	" 75.55, " 10.43
		" 75.44, " 10.47

This compound, when mixed with Compound A27, m.p. 199–200°, showed a depression in melting point to 160–180°. When mixed with Compound A32, m.p. 192–194°, the mixture melted at 165–172°.

The diacetate of allopregnanediol-3α,6-one-20 was prepared by heating 25 mg. under a reflux for 1 hour in 1 ml. of pyridine and 0.5 ml. of acetic anhydride; it was recrystallized from ligroin as needles melting at 155–156°; $[\alpha]_D^{24} = +92.3° \pm 2°$ (10.01 mg. in 2.98 ml. of ethanol).

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[10] We are grateful to Dr. H. L. Mason for calling our attention to the incorrect specific rotation of Compound A30 reported previously (4). The rotation has been repeated and was found to be $[\alpha]_D^{25} = +64.5° \pm 4°$ (4.96 mg. in 2.00 ml. of ethanol).

Analysis—$C_{25}H_{38}O_5$. Calculated, C 71.74, H 9.15; found, C 71.89, H 9.37

The monoxime prepared in the usual way was recrystallized from methanol and melted at 288–291°.

Analysis—$C_{21}H_{35}O_3N$. Calculated, N 4.01; found, N 4.19

The monodinitrophenylhydrazone melted at 235° after crystallization from methanol-water.

Analysis—$C_{27}H_{38}O_6N_4$. Calculated. C 63.02, H 7.44, N 10.88
Found. " 62.66, " 7.51, " 10.71

The dihydroxy ketone was recovered unchanged by heating 25 mg. in 0.3 ml. of hydrochloric acid in acetic acid (1:9 by volume), nor was it oxidized by periodic acid in methanol at room temperature. The structure was established by the following reactions. Oxidation with sodium hypoiodite gave iodoform and an acid which melted at 278–282°. Oxidation with chromic acid yielded a triketone which melted at 235–237° after crystallization from ethyl acetate-ligroin. $[\alpha]_D^{25} = +47.2° \pm 9°$ (4.42 mg. in 2.98 ml. of ethanol).

Analysis—$C_{21}H_{30}O_3$. Calculated, C 76.32, H 9.15; found, C 76.06, H 9.03

The triketone did not depress the melting point of allopregnanetrione-3,6,20, m.p. 235–240°, $[\alpha]_D^{24} = +43.3° \pm 6°$ (3.24 mg. in 2.00 ml. of ethanol), which was prepared from Δ^5-pregnenol-3β-one-20 by the method described by Marker *et al.* (71).

The trisdinitrophenylhydrazone was recrystallized from chloroform and methanol, melting at 270–275°, and did not depress the melting point of the trisdinitrophenylhydrazone prepared from this synthetic sample.

Compound A32. 192–194°—About 140 mg. of this compound were obtained from a pool of 1000 gallons of human pregnancy urine (Case PP1). It was eluted from alumina with 2 per cent methanol in ether and was isolated from the benzene and acetone-ether mother liquors of Compound A31, m.p. 195–196°. When benzene was added to a solution of this compound in acetone, rosettes of needles crystallized; this crystal form melted unsharply between 95–105°. When the substance was recrystallized from acetone alone or from acetone-isopropyl ether mixtures, needles were obtained which melted at 192–194°; $[\alpha]_D^{24} = +66.3° \pm 3°$ (10.34 mg. in 2.98 ml. of ethanol). It depressed the melting point of Compound A31, m.p. 195–196°, and also of Compound A27, m.p. 199–200°.

Analysis—$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10.25; found, C 75.53, H 10.27

It formed a diacetate melting at 128–129° after recrystallization from acetone-ether mixtures.

Analysis—$C_{25}H_{38}O_5$. Calculated, C 71.74, H 9.15; found, C 71.69, H 9.07

It formed a dinitrophenylhydrazone melting at 234–238° after recrystallization from methanol, which depressed the melting point of the monodinitrophenylhydrazone of Compound A31.

Compound A33. 230–233°—Traces of this compound were isolated from the urine of a male eunuch with adrenal cortical hyperplasia (Case AHM4) and from the urine of a man with cancer of the prostate (Case CaP1). It was eluted from alumina with acetone-ether (1:3) and was recrystallized from acetone as needles melting at 230–233°. When mixed with Compound A34, m.p. 238–241°, the mixture melted at 210–220°, and when mixed with Compound A29, m.p. 235–237°, it melted at 185–220°.

Compound A34. 238–241°—Traces of this compound were obtained from the urine of a male eunuch with adrenal cortical hyperplasia (Case AHM4). It was eluted from alumina with acetone-ether (1:4). When mixed with Compound A33, m.p. 230–233°, it melted at 210–220°, and when mixed with Compound A29, m.p. 235–237°, it melted at 206–214°.

Compound A35. 172–176°—This substance was isolated from the urine of a male eunuch with adrenal cortical hyperplasia (Case AHM4) and from the urine of a woman with adrenogenital syndrome due to an adrenal cortical hyperplasia (Case AHF2). It was eluted from alumina with acetone. When recrystallized from acetone, it formed needles melting at 172–176° which depressed the melting point of Compound A26, m.p. 174–175°.

β-Ketosteroids

Compound B1. $\Delta^{2\ (\text{or}\ 3)}$-Allopregnenone-20 (3)—This compound was isolated for the first time from a pool of human pregnancy urine (Case PP1) and from the urine of a woman with adrenogenital syndrome due to an adrenal cortical tumor (Case ATF1). It was eluted from alumina with ether-benzene (1:10) mixture. When recrystallized from aqueous methanol, long needles formed which melted at 127–129°; $[\alpha]_D^{25} = +132° \pm 4°$ (7.67 mg. in 2.98 ml. of ethanol).

Analysis—$C_{21}H_{32}O$. Calculated, C 83.91, H 10.75; found, C 83.89, H 10.87

It formed a semicarbazone, which, when recrystallized from absolute ethanol, melted at 260° with decomposition. In chloroform solution, it gave a yellow color when treated with tetranitromethane. Although this compound possesses no hydroxyl groups, it reacted with a 1 per cent digitonin solution to give an insoluble digitonide. There was no depression in melting point when mixed with an authentic sample of $\Delta^{2\ (\text{or}\ 3)}$-allopregnenone-20, prepared by the following procedure: Allopregnanol-3α-one-20 was dissolved in a mixture of pyridine and chloroform and treated with chlorosulfonic acid. After standing 1 day at room temperature, the solution was concentrated to dryness *in vacuo*. Ether was added to the residue and after cooling the pyridinium salt was filtered. It was dissolved in methanol and neutralized with 5 per cent methanolic potassium hydroxide. Ether was added to the cooled solution and the precipitated potassium allopregnanolone sulfate was filtered. It was recrystallized from ethanol and melted at 207–210°.[11] The sulfate was pyrolyzed at 250°, in a sublimation apparatus at 10^{-5} mm. pressure. The crystalline sublimate was recrystallized first from dilute methanol and then from methanol to give long needles melting at 125°. When mixed with the sample obtained from urine, the mixture melted at 125–127°; $[\alpha]_D^{25} = +133° \pm 4°$ (7.65 mg. in 2.98 ml. of ethanol).

The synthetic sample was sublimed in high vacuum for analysis.

Analysis—$C_{21}H_{32}O$. Calculated, C 83.91, H 10.75; found, C 84.43, H 10.80

Compound B2. Allopregnanol-3β-one-20—This compound was isolated from a pool of human pregnancy urine (Case PP1), in which it had been found previously (72). It was eluted from alumina with ether-benzene (1:9) mixture, and crystallized as plates from aqueous methanol, m.p. 194–195°; $[\alpha]_D^{27} = +91.2°$ (11.77 mg. in 2.98 ml. of ethanol). When mixed with an authentic sample of allopregnanol-3β-one-20, there was no depression in the melting point. Butenandt and Mamoli (51) reported a melting point of 194°; $[\alpha]_D^{19} \doteq +90.8°$ (in ethanol).

Analysis—$C_{21}H_{34}O_2$. Calculated, C 79.19, H 10.76; found, C 79.20, H 10.80

The acetate melted at 144–146°. It did not depress the melting point of an authentic sample.

Compound B3. Isoandrosterone—This compound has been isolated from the urine of normal women with virilism (42, 30, 73, 74), from the urine of two women and from the urine of men with cancer (42, 73). In the investigation herein reported it was found in the urine of normal men and women, of a pregnant woman (Case P1), of two women with cancer of the breast (Cases CaBF10 and CaBF21), and of a eunuchoid male following testosterone administration (Case EMIX1). It was eluted from alumina with ether-benzene (1:2) mixture. Crystallization from aqueous methanol gave a sample melting at 175–176°, $[\alpha]_D^{24} = +82.0° \pm 9.8°$ (3.63 mg. in 2.98 ml. of methanol), which did not depress the melting point of an authentic specimen. Ruzicka, Goldberg, and Brüngger (75) reported a melting point of 174–174.5°; $[\alpha]_D = +87.5°$ (methanol).

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[11] Potassium pregnanol-3α-one-20 sulfate prepared in a similar manner melted at 232-234°. When pyrolyzed in a high vacuum, it yielded $\Delta^{2(\text{or } 3)}$-pregnenone-20, m.p. 128–130°; $[\alpha]_D^{24} = +94.5° \pm 9°$ (in ethanol).

Analysis—$C_{19}H_{30}O_2$. Calculated, C 78.57, H 10.40; found, C 78.29, H 10.07

The benzoate melted at 210–211° and did not depress the melting point of an authentic sample.

Compound B4. Dehydroisoandrosterone—This compound has been isolated from the urine of normal human subjects (43, 76, 77), of normal men (42, 56), of normal women (57), of male castrates (59), of female castrates (44), of patients with adrenal cortical tumors (31, 41, 78), and of patients with adrenal cortical hyperplasia (31, 30). In the investigation herein reported this compound was isolated from the urine of all patients with adrenal cortical hyperplasia and adrenal cortical tumors and of almost every subject investigated. It was eluted from alumina with ether-benzene (1:3) mixture. Recrystallization from benzene-ligroin mixtures or aqueous methanol gave dimorphous crystals melting at 137–138° and at 145–146°. There was no depression in the melting point when Compound B4 was mixed with an authentic sample. The analytical sample was sublimed in high vacuum. $[\alpha]_D^{25} = +10.4°$ (17.18 mg. in 2.98 ml. of ethanol). Butenandt *et al.* (76) reported a melting point of 148°; $[\alpha]_D^{18} = +10.9° \pm 0.7°$ (ethanol).

Analysis—$C_{19}H_{28}O_2$. Calculated, C 79.12, H 9.78; found, C 79.11, H 10.05

The acetate recrystallized from dilute methanol melted at 166–166.5° and the benzoate recrystallized from acetone melted at 256–257°. Neither of these derivatives depressed the melting point of authentic samples.

Compound B5. 272–275°—This substance was isolated in traces from a pool of human pregnancy urine (Case PP1) and from the urine of a woman with adrenogenital syndrome due to cortical tumor (Case ATF1). It was eluted from alumina with 1 per cent methanol in ether and from magnesium silicate-Celite (1:2 by weight) with ether-benzene (1:5) mixture. It was eluted before Compound B6. Recrystallization from acetone gave needles melting at 272–275° (uncorrected).

Compound B6. 238–240°—This compound has been found in traces in the urine of three women with adrenogenital syndrome due to an adrenal cortical hyperplasia (Cases AHF2, AHF10, and AHF13), of two women with adrenogenital syndrome due to an adrenal cortical tumor (Cases ATF1 and ATF2), of a male eunuch with adrenal cortical hyperplasia (Case AHM4), and of a woman with a breast cancer (Case CaBF21). It was eluted from alumina with 2 per cent methanol in ether and from magnesium silicate-Celite with ether-benzene mixture (1:5). This substance is altered by chromatographing on alumina and yields non-crystallizable oils. It appears likely, therefore, that larger quantities of the compound were excreted but were not isolated. This compound was recrystallized from dilute ethanol, acetone-ligroin, or benzene-ligroin and melted at 238–240°.

Analysis—$C_{21}H_{30}O_3$. Calculated, C 76.33, H 9.14; found, C 75.87, H 8.98

The ultraviolet absorption spectrum, kindly determined by Dr. Douglas Bowen, showed an extinction coefficient $\log \epsilon = 4.07$ at 237.3 mμ, characteristic of an α,β-unsaturated carbonyl group. When acetylated with pyridine and acetic anhydride at room temperature a product was obtained, which, when recrystallized from ligroin, melted at 175–176°. This acetoxy derivative likewise exhibited strong absorption in the ultraviolet region at 234 mμ.

Compound B7. 209–210°—This compound was obtained from a pool of human pregnancy urine (Case PP1). It was eluted from alumina with 2 per cent methanol in ether. When recrystallized from aqueous methanol, long needles were obtained which melted at 209–210°; $[\alpha]_D^{25} = +98.8° \pm 2°$ (16.59 mg. in 2.98 ml. of ethanol).

Analysis—$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10.25; found, C 75.55, H 10.23

The diacetate was recrystallized from dilute methanol, m.p. 102.5–103.5°.

Analysis—$C_{25}H_{32}O_5$. Calculated, C 71.74, H 9.15; found, C 71.65, H 9.25

The monoxime was crystallized from dilute ethanol and melted at 275° with decomposition.

Analysis—$C_{21}H_{35}NO_3$. Calculated, C 72.16, H 10.10; found, C 71.88, H 10.10

Oxidation of the compound with chromium trioxide gave a product melting at 236–237°, which was different from that of the triketone, allopregnanetrione-3,6,20 obtained from the isomeric α-ketosteroid, Compound A31, m.p. 195–196°.

SUMMARY

1. The chemical characteristics of *thirty-five* α-ketosteroids and *seven* β-ketosteroids that have been isolated from human urine are described.

2. *Twenty-six* of these forty-two steroids have been completely identified. This group includes (*a*) *fourteen* that have not been isolated previously from human urine, of which *ten* are known compounds: etiocholanol-3α-one-17 acetate-3; $\Delta^{11(?)}$-androstenol-3α-one-17 acetate-3; androsterone acetate; allopregnanedione-3,20; pregnanedione-3,20; androstanedione-3,17; etiocholanedione-3,17; Δ^4-androstenedione-3,17; 17-isopregnanol-3α-one-20; and etiocholanol-3α-dione-11,17; and *four* are new steroids: Δ^9-etiocholenol-3α-one-17; pregnanediol-3α,17α-one-20; allopregnanediol-3α,6-one-20; and $\Delta^{2\ (\text{or}\ 3)}$-allopregnenone-20; and (*b*) *twelve* that have been isolated

before from human urine by other investigators: $\Delta^{3,5}$-androstadienone-17; $\Delta^{2\ (\mathrm{or}\ 3)}$-androstenone-17; 3-chloro-Δ^{5}-androstenone-17; allopregnanol-3α-one-20; pregnanol-3α-one-20; androsterone; Δ^{9}-androstenol-3α-one-17; etiocholanol-3α-one-17; androstanediol-3α,11β-one-17; allopregnanol-3β-one-20; isoandrosterone; and dehydroisoandrosterone.

3. *Sixteen* of these forty-two steroids have been incompletely identified. This group includes *six* that have been characterized by melting point and C and H analysis, and *ten* that were isolated in such small amounts that they have been characterized only by melting points.

4. The sequence in which these steroids are eluted from aluminum oxide and magnesium silicate chromatographic columns is discussed; some general principles concerning the order of elution are presented.

5. The relationships of the steroids isolated as metabolites in urine to the known and postulated precursors among the hormones are discussed; certain deductions concerning these relationships are given.

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STUDIES IN STEROID METABOLISM

III. THE APPLICATION OF INFRA-RED SPECTROMETRY TO THE FRACTIONATION OF URINARY KETOSTEROIDS*

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It has been recognized for some considerable time that the near infra-red absorption spectrum is one of the most highly characteristic physical properties of a molecule, and that the measurement of the infra-red absorption spectrum provides a very sensitive method for the identification of organic compounds. Infra-red absorption spectra until recently have been determined in relatively few laboratories, and the data have been used mainly in connection with studies leading to the determination of the interatomic force constants and the geometric structure of simple molecules (4). More recently the infra-red method has been applied to certain industrial analytical problems (5–8).

It is to be expected that the technique will soon be used extensively in medical and biological research, since adequate instruments are now available commercially. In this paper is described the use of infra-red spectrometry as an analytical tool for the detection and characterization of small quantities of steroid metabolites in urinary fractions. The infra-red spectra of certain steroids have been reported recently (9, 10).

The problem involved has been outlined in earlier publications (1–3, 11, 12). Briefly it is concerned with an analysis and comparative study of the steroid content of human urine in normal and pathological states. Infra-red analysis has been applied to the crude mixtures of urinary ketosteroids and, more extensively, to the materials obtained by the systematic chromatographic analysis of these mixtures. These procedures have been described in Papers I and II (11, 12), which deal with the methods of separation, isolation, and chemical identification of ketosteroids present in urine.

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EXPERIMENTAL

Methods

Determination of Infra-Red Spectra—A Perkin-Elmer model 12a infra-red spectrometer (13) with sodium chloride prism was used; the thermoelectric current was fed into a high sensitivity galvanometer (Leeds and Northrup, 0.17 microvolt per mm.), the mirror deflections of which were amplified by means of a photoelectric relay (7) and secondary galvanometer (Leeds and Northrup, 0.003 microampere per mm.). The deflections of the secondary galvanometer were recorded automatically on a strip chart recorder[1] of the type described by Barnes, Gore, Liddel, and Williams (7, 14). The recorder drive was operated by a constant speed motor, while a three speed gear box in the drive mechanism of the Littrow mirror allowed for variation in the speed of recording the spectrum. This apparatus was housed in a laboratory air-conditioned to maintain a temperature of 24° ± 1° and a relative humidity of less than 40 per cent. The sensitivity of the instrument was checked daily by the determination of the absorption of a standard sample of ammonia vapor over the range between 800 and 1200 cm.$^{-1}$. This served also as a check on the frequency scale calibration, although, as the positions of the absorption bands were matched by the use of external standards in a manner described below, such calibration was of secondary significance for the empirical comparison of the spectra which formed the bulk of the work reported here.

The samples were analyzed as solutions in carbon disulfide in cells made from two rectangular blocks of sodium chloride (14 × 20 mm.) separated by brass spacers 1 mm. thick.[2] The sodium chloride plates were sealed together with Glyptal cement and baked at 100° overnight. Since the analyses were qualitative only, the exact thicknesses of the cells were not determined; two cells were used alternately, one being cleaned and filled, while a measurement was made on a solution in the other. The cells were filled through two holes (3 mm. diameter) drilled through the top of the spacer, which was widened for the purpose. When the cells were in use, these holes were closed by tapered brass pegs to prevent evaporation of the solvent. The solutions were introduced and removed from the cells by means of capillary pipettes fitted with rubber bulbs. The cells were cleaned by repeatedly flushing with carbon disulfide. Because of the inflammability and toxicity of this solvent, special care had to be taken during these manipulations; solvent residues were not allowed to accumulate in the laboratory, and the cells were drained by attachment to a vacuum line so that the carbon disulfide evaporated rapidly and was carried into the vacuum system with a current of air. The choice of solvent is limited by the fact that very few liquids are sufficiently transparent to infra-red radiation over an extensive spectral region. Of the few, carbon disulfide is best in the region of particular study, 1180 to 875 cm.$^{-1}$.

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[1] An electronic amplifier and recorder is now available for use with the spectrometer in place of this galvanometer method of recording, and both types of equipment have been employed in the later phases of this work.

[2] A 3 mm. cell was used in certain instances in which the material was not readily soluble in carbon disulfide.

Observations, both on pure crystalline steroids and on urinary fractions, have shown that a concentration of 0.5 to 2 per cent of solute by weight is needed to give a good spectrum in a 1 mm. layer of carbon disulfide. The volume of solution needed in the cell was 0.2 ml., from which it may be observed that a minimum of between 1 and 4 mg. of material is required for an infra-red analysis.[3]

TABLE I

Regions of Spectrum Examined

Region	Frequency	Wave-length
	cm.$^{-1}$	*μ*
A	4000–2530	2.50– 3.95
B	2000–1620	5.00– 6.17
C	1315–1150	7.60– 8.69
D	1180– 875	8.47–11.43

While most of the fractions were examined over the frequency[4] range between 1180 and 875 cm.$^{-1}$, many of the samples were examined also in other regions of the spectrum (see Table I). For purposes of discussion these various spectral ranges will be referred to as Regions A, B, C, and D respectively.

The spectrographic record obtained from the particular strip chart recorder employed did not have frequency or wave-length markings, and it was necessary to provide suitable calibration points at known frequencies in order that the relative positions of absorption bands on different charts might be compared. In Region A the atmospheric water vapor absorption bands at 3741 and 3882 cm.$^{-1}$ are present on all curves and serve as calibration points (Fig. 1); likewise the atmospheric water vapor bands at 1700, 1830, and 1870 cm.$^{-1}$ provide calibration points in Region B (Fig. 2). For Region C the water vapor bands at 1388, 1396, and 1420 cm.$^{-1}$ were employed as calibration points (see Curve IV, Fig. 3) in the manner described

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[3] In the case of urinary fractions, the dilution required to yield a solution within the necessary concentration range was calculated in terms of the "mg.-equivalent of androsterone" as determined colorimetrically by the Zimmermann reaction, and not from the actual weight of the fraction (11).

[4] The units most often used in designating portions of the infra-red spectrum are wave-length unit, micron or μ (1 μ = 10,000 A = 10^{-4} cm.), and the so called frequency unit, wave number or cm.$^{-1}$ [1 cm.$^{-1}$ = 1/λ (cm.)].

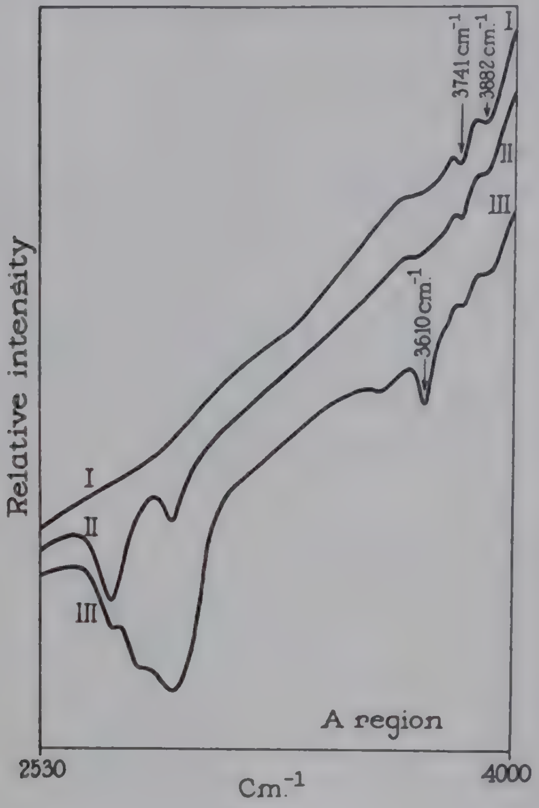

FIG. 1

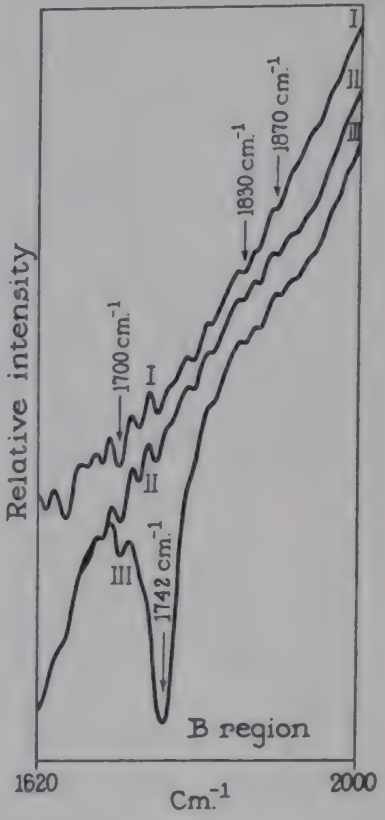

FIG. 2

FIG. 1. Region A (4000 to 2530 cm.$^{-1}$); reproduction of a typical record obtained directly from the spectrometer. Note that this is not a per cent transmission curve. Curve I, radiation with bands due to atmospheric water vapor; Curve II, carbon disulfide; Curve III, solution of androsterone in carbon disulfide. The atmospheric water vapor absorption bands at 3741 and 3882 cm.$^{-1}$ are present on all curves and serve as calibration points. The absorption band at 3610 cm.$^{-1}$ in the androsterone curve (No. III) is associated with the longitudinal vibration in the hydrogen-oxygen bond of the hydroxyl group.

FIG. 2. Region B (2000 to 1620 cm.$^{-1}$); reproduction of a typical record obtained directly from the spectrometer. Curve I, radiation with bands due to atmospheric water vapor; Curve II, carbon disulfide; Curve III, solution of androsterone in carbon disulfide. The atmospheric water vapor bands at 1700, 1830, and 1870 cm.$^{-1}$ provide calibration points in all three curves. The band at 1742 cm.$^{-1}$ in the androsterone curve (No. III) is related to the longitudinal vibrations in the carbon-oxygen bond of the carbonyl group.

below for Region D. In Region D, the "solvent control curve" obtained in the absence of absorbing material in the cell is smooth (Curve II, Fig. 4), and in order to provide reference frequencies the absorption bands of acetone vapor at 1230, 1219, and 1207 cm.$^{-1}$ were superimposed on each experimental curve (Curve VIII, Fig. 4). This was done by starting spectral recording at a frequency of about 1250 cm.$^{-1}$ with a 10 mm. cell containing air saturated with acetone vapor in the light path. The spectrum was recorded to about 1185 cm.$^{-1}$, where the instrument was stopped, the sample cell inserted in place of the acetone vapor cell, and the instrument started again. During this exchange of cells care was taken that no adjustments were made to the drive mechanism of the Littrow mirror so that any error due to backlash in the drive mechanism was minimized.

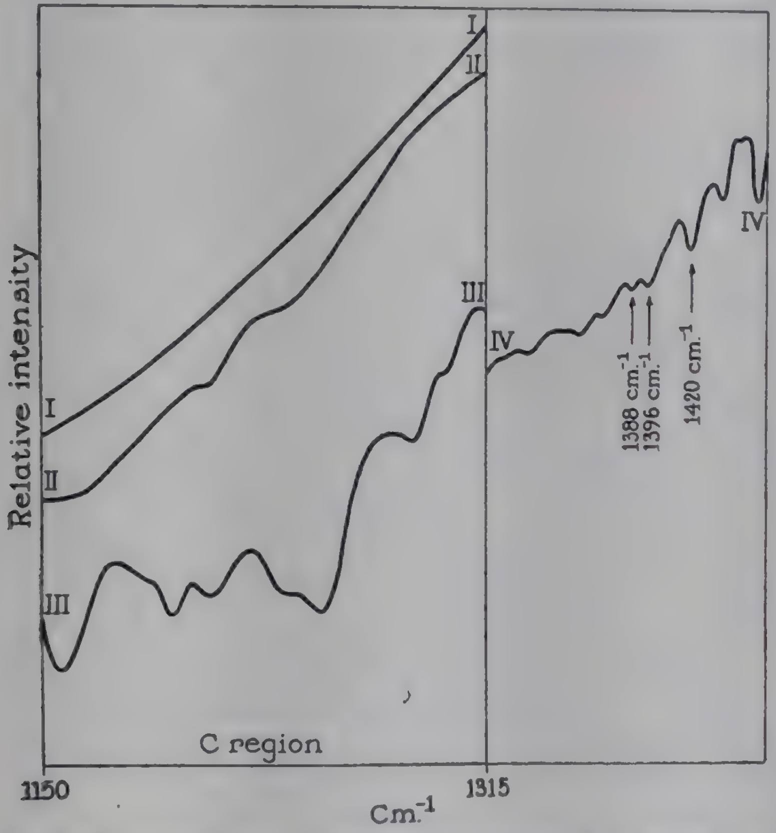

FIG. 3. Region C (1315 to 1150 cm.$^{-1}$); reproduction of a typical record obtained directly from the spectrometer. Curve I, radiation; Curve II, carbon disulfide; Curve III, solution of androsterone in carbon disulfide; Curve IV, calibration spectrum of water vapor between 1460 and 1315 cm.$^{-1}$. Since there are no absorption bands (Curve I) to serve as reference points in this region, the water vapor absorption bands (Curve IV) of the region between 1460 and 1315 cm.$^{-1}$ are used as reference points.

Calculation of Data

The curve obtained in this way represents the absorption of the sample superimposed on that of the solvent and of the atmospheric water vapor

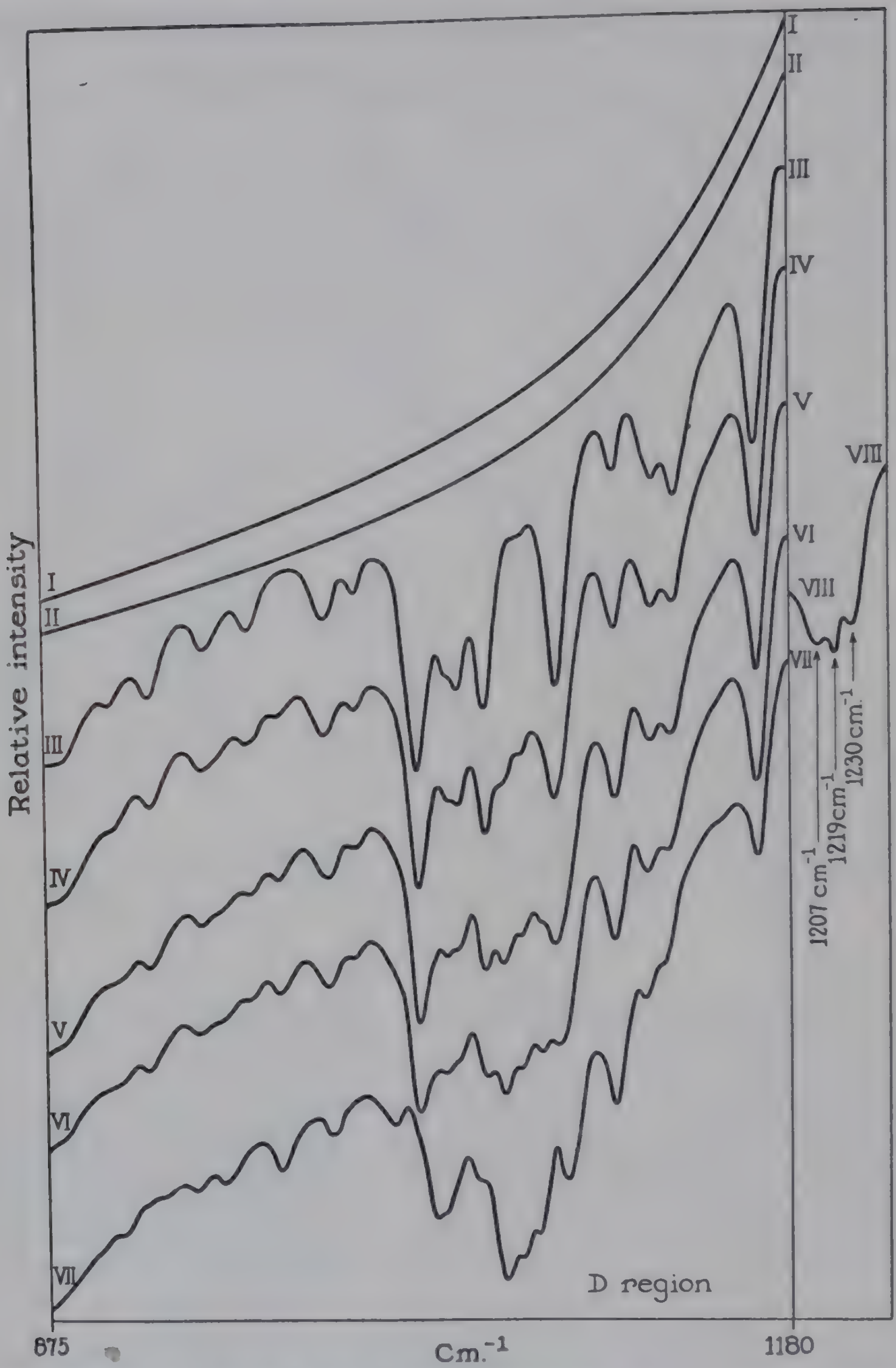

FIG. 4. Region D (1180 to 875 cm.$^{-1}$); reproduction of a typical record obtained directly from the spectrometer. Curve I, radiation; Curve II, carbon disulfide; Curve III, solution of androsterone in carbon disulfide; Curve IV, 75 per cent androsterone + 25 per cent etiocholanol-3α-one-17; Curve V, 50 per cent androsterone + 50 per cent etiocholanol-3α-one-17; Curve VI, 25 per cent androsterone + 75 per cent etiocholanol-3α-one-17; Curve VII, solution of etiocholanol-3α-one-17; Curve VIII, calibration spectrum of acetone vapor between 1250 and 1180 cm.$^{-1}$. Since there are no absorption bands (Curve I) to serve as reference points in this region, the acetone

and carbon dioxide in the optical path. Even in the absence of any absorbing material in the light path, the record obtained from the instrument would be a sloping line, since the energy measured by the thermocouple varies as the frequency changes. The energy radiated by the light source is approximately that of a black body at 1400°. For a given slit width, the energy incident on the thermocouple is dependent both on the shape of this black body radiation curve and on the optical characteristics of the monochromator system. The energy is maximal for a frequency of about 4000 cm.$^{-1}$, and in the regions covered by these investigations the energy decreases with decreasing frequency.

In reporting the spectra of pure substances, it is customary to calculate either a per cent-transmission or a per cent-absorption curve in which corrections are applied for the absorption of the solvent, the atmosphere, and the slope of the radiation line. The calculations involved in the preparation of such per cent-absorption curves from the instrumental records are tedious and time-consuming,[5] and it has been found that, for the empirical comparison of the spectra of urinary fractions, the records obtained directly from the strip chart recorder (referred to as instrumental curves) can be used quite satisfactorily provided that the instrumental conditions are carefully standardized. Such conditions are the slit width, the power consumption of the light source, the speed of rotation of the recorder drum, the speed of rotation of the Littrow mirror, the amplification of the thermoelectric current, and the direction in which the spectrum is determined (from high to low frequency). This careful standardization of operating conditions and the consequent elimination of the need for the calculation of the per cent-absorption curves of the urinary fractions make the method sufficiently

[5] In recording the ultraviolet absorption spectrum of a pure organic compound, it is customary to transform the intensity measurements as experimentally determined into molecular extinction coefficients and to report the results in the form of a curve with molecular extinction coefficients as ordinates and wave-lengths or frequencies as abscissae. In the infra-red region experimental difficulties caused mainly by the presence of scattered radiation, the variation in slit widths employed, and the uncertainties in determining sample thickness accurately make it undesirable at the present time to express intensity data in absolute units. The per cent-absorption refers only to the particular sample determined on a particular instrument. While, in general, such measurements are reproducible, the spectra of pure compounds as measured on different instruments may show variations in the relative intensity of the maxima in different regions of the spectrum.

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vapor absorption bands (Curve VIII) between 1250 and 1180 cm.$^{-1}$ are used as reference points. The curves (Nos. III to VII) of androsterone and etiocholanol-3α-one-17 and of the three binary mixtures of these steroids exhibit distinctive variations from each other. Since the greatest difference in absorption between steroids is found in Region D, this region is employed for identification purposes.

rapid to be applied as a practical tool in conjunction with chromatographic analysis. At the same time, it must be pointed out that data so obtained can be directly compared only with other data obtained on the same instrument or on a second instrument carefully matched for the purpose. In the recording of the spectra of pure substances, such as are discussed in the following section, the per cent-absorption curves are calculated, since these contain less instrumental variations and approach more closely to the absolute physical constants of the individual substances.

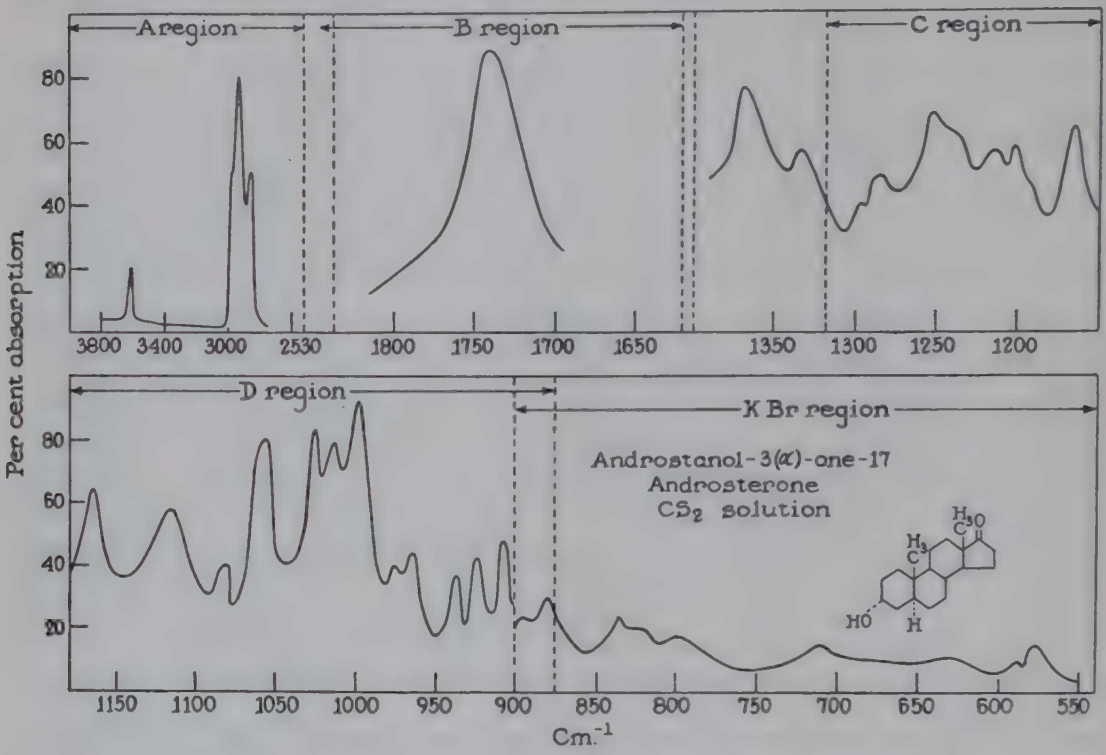

Fig. 5. Infra-red absorption spectrum of androsterone

Results

Infra-Red Spectra of Pure Compounds—As typical examples of the infrared absorption spectra of pure steroids in carbon disulfide solution, the (per cent-absorption)/(frequency) curves of the following four stereoisomers are illustrated in Figs. 5 to 8: I, androstanol-3α-one-17 (androsterone); II, androstanol-3β-one-17 (isoandrosterone); III, etiocholanol-3α-one-17 (α-etiocholanolone); IV, etiocholanol-3β-one-17 (β-etiocholanolone).

The four regions covered in the routine analyses of urinary fractions are indicated by the dotted ordinate lines.[6] At the high frequency end of the spectrum (Region A) the absorption bands are related to energy absorption in the molecule associated with activation of stretching vibrations of certain of the bonds; thus the absorption bands at 3610 cm.$^{-1}$ in androsterone and its three isomers (Figs. 5 to 8) are associated with a longitudinal vibration in the hydrogen-oxygen bond of the hydroxyl group. Similarly the bands at 1742 cm.$^{-1}$ in the four compounds are related to longitudinal vibrations in the carbon-oxygen bond of the carbonyl group. It is at the lower frequencies (Region D) that the differences between the spectra of the various steroids are most significant; this is because the absorption of radiation in this region is more nearly characteristic of vibrations involving the whole or extensive parts of the molecule, and such vibrations are more sensitive to small variations in the molecular configuration.

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[6] The region between 1400 and 1315 cm.$^{-1}$ shown on Figs. 5 to 8 is excluded from Region C because it occurs on the side of a steeply rising carbon disulfide absorption band which makes measurements here unsuitable for empirical comparison without

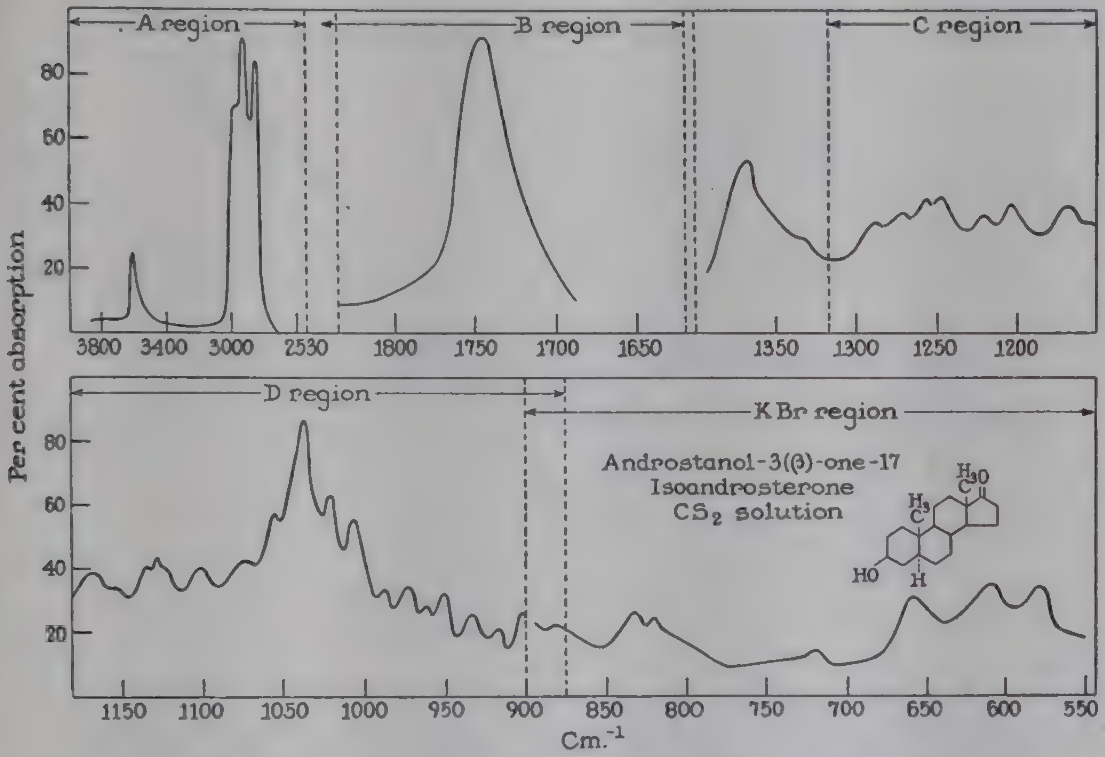

FIG. 6. Infra-red absorption spectrum of isoandrosterone

Accordingly, it is to Region D that most attention has been directed in these empirical comparisons and analyses of the spectra of urinary frac-

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correction for the carbon disulfide absorption. The "*K*Br region" measurements included in these curves were determined at the Stamford Research Laboratories, American Cyanamid Company, by means of a spectrograph with a potassium bromide prism. The data for Region A of Figs. 5 to 8 were also determined at Stamford with a lithium fluoride prism. The concentrations and cell thicknesses employed in the measurements in these regions of the spectrum were not necessarily identical with those used for Regions B, C, and D.

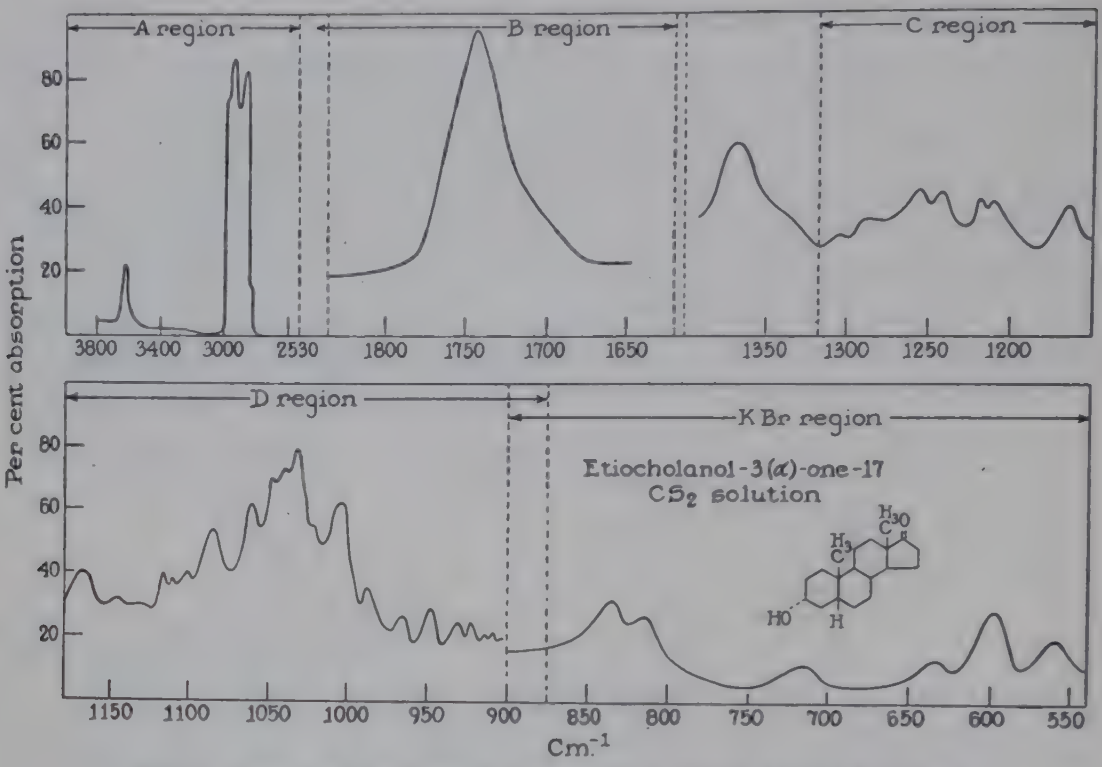

FIG. 7. Infra-red absorption spectrum of etiocholanol-3α-one-17

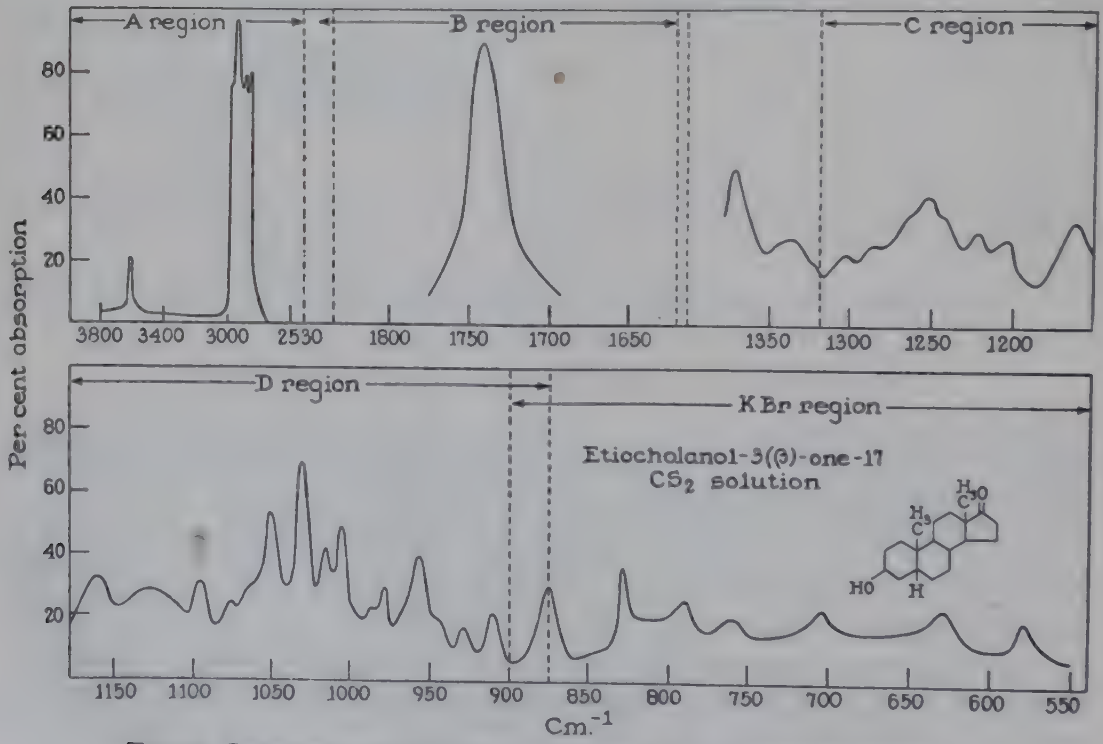

FIG. 8. Infra-red absorption spectrum of etiocholanol-3β-one-17

tions. In some cases, however, differences in Regions A, B, and C have proved of value in identifying the presence of certain characteristic group-

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ings. The spectra of a considerable number of pure steroids have been determined, and in all cases marked individuality is noted in Region D. Recently the infra-red spectra of crystalline films of several steroids have been reported by Furchgott, Rosenkrantz, and Shorr (9, 10), who have also made an attempt to correlate certain of the absorption maxima with specific groups and their stereochemical relations in the molecule.

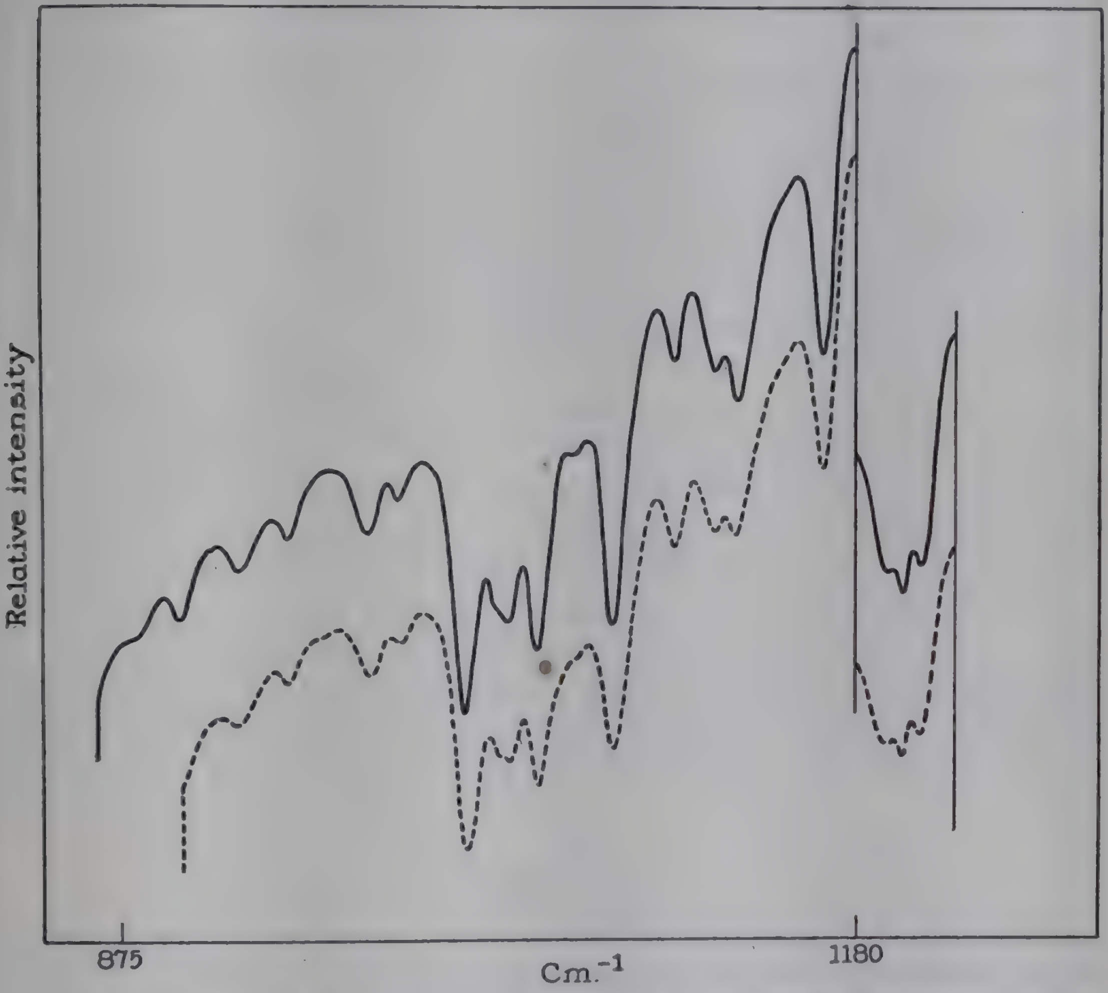

Fig. 9. Photograph of the graphic comparison in Region D of the curve of pure androsterone with that of a non-crystalline steroid isolated from urine and presumed from its spectrum to be androsterone. Curve with solid line, androsterone standard; curve with dotted line, steroid from urine fraction.

Spectra of Mixtures of Pure Steroids—The infra-red absorption spectra of mixtures should show structures indicative of the presence of the various constituents, and, in the absence of intermolecular reactions, the spectrum of a mixture of two or more organic compounds should be a summation of the spectra of the constituents, proper allowance being made for their respective concentrations.[7]

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[7] The optical density (log I_0/I), where I_0 is the intensity of the incident radiation and I the intensity of the transmitted beam, is directly proportional to the concen-

The quantitative analysis of several mixtures of two or more organic compounds, based on measurements of the infra-red absorption spectra, have been recorded in the literature (5, 7). The spectra of a series of mixtures of androsterone and etiocholanol-3α-one-17 in Region D are shown in Fig. 4. The systematic spectral variation with change in composition is clearly evident in these curves, and approximate quantitative analyses of mixtures of androsterone and etiocholanol-3α-one-17 can be made by visual matching of such an experimental curve against a range of control curves made on mixtures of known composition. For such purposes the direct instrumental curves obtained under controlled conditions of operation will suffice and per cent-absorption curves are not necessary.

Spectra of Urinary Fractions—The work carried out at the Sloan-Kettering Institute (11, 12) has shown that the ketosteroid mixture obtained from urines of normal and diseased persons is very complex; the infra-red analyses of these crude fractions support this view.

If this crude ketonic material is submitted to a process of systematic fractional adsorption analysis, the infra-red absorption spectra of the successive fractions are found to exhibit considerable differences. Certain of these fractions obtained from such a chromatographic analysis yield infra-red curves which are identical with those of pure constituents of established structure; this is illustrated in Fig. 9, in which the instrumental curve in Region D of a fraction from the urine of a normal person is compared with the curve of pure androsterone. Among the products of chromatographic separation of the crude ketosteroid material, forty-two pure compounds have been identified by the established methods of organic chemistry (12). The spectrum of each of these constituents is characteristic.

DISCUSSION

An empirical comparison of the instrumental curves, obtained from a chromatographic sequence of urinary fractions, with a set of standard curves prepared from pure samples of the known constituents clearly demonstrates that the presence of such constituents can be detected in the individual fractions. In making use of infra-red absorption data in this manner, it is assumed that each steroid metabolite possesses a unique spectrum and can be recognized by this in the analytical procedure. Observations of the spectra of a considerable number of steroids have shown that quite minor changes in chemical structure or stereochemical configuration alter the spectrum significantly, and there is ample evidence from the recorded

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tration of the solute. Hence, in the spectrum of a mixture, the heights of the maxima will be directly proportional to the concentrations only if the absorption intensity data are expressed as optical densities.

data on the infra-red spectra of other types of compounds (6, 7, 15) to justify the assumption that the infra-red spectrum of every organic compound is probably unique. These conclusions are further strengthened by a study of the spectra of Figs. 5 to 8. It is evident that these spectra differ widely, even though the four compounds differ only in their stereoisomerism.

In such applications infra-red analysis has several advantages besides the unique character of the spectrum. As shown in the experimental section, the sample required is small, and can be reduced still further by special optics or the design of micro cells. The sample is unchanged in the analysis and can be recovered for further work. The time required to record Region D, including the preparation of the sample, is less than 10 minutes, and the spectral record can be obtained in a permanent form by relatively untrained personnel. Trained specialists are thus required only for interpretation of the records at convenient time intervals. Further development of the technique may permit the use of smaller urine collections and less tedious chromatographic separation.

In addition to the application of infra-red spectroscopy as a means of empirical identification of steroids, the use of these spectra to elucidate the chemical structure of steroids is possible, because of the correlations that have been made between the infra-red absorption spectra and the chemical structure of steroid molecules. It has been noted that certain of the absorption bands in the infra-red spectrum are associated with the presence of hydroxyl and carbonyl groups in the molecule. In the work discussed here, no attempt is made to deduce structural information from the characteristic absorption bands of the spectra. However, it is clear that infra-red data can be applied to the elucidation of the structure of unidentified steroids isolated from urine.

The chromatographic separations yield a large number of fractions, many of which contain insufficient material to allow further characterization by chemical methods; this is true particularly of the non-crystalline fractions, and the infra-red absorption spectra provide a means for the characterization of such material. If the spectra of a consecutive group of fractions from the chromatographic column are followed, those fractions which consist of one only of the previously recognized constituents can be identified by comparison of the experimental curves with those of the known standards. The position, in the series of consecutive fractions, in which a second constituent approaches a significant concentration can be recognized by the progressive deviation of the appearance of the experimental curve from that of the pure reference compound or compounds.

If the eluates from the chromatogram are systematically followed in this manner, variations in the spectra appear at certain places which do not correspond with those which would be anticipated (11, 12) for binary mixtures of the reference compounds in the well established sequence in which they are eluted from the chromatographic column. The presence of such anomalous structure in a spectrum indicates that the fraction contains material not hitherto recognized as a constituent of these steroid mixtures. The presence of small quantities of several previously undetected steroid metabolites in the urines of normal and diseased persons has been indicated in this manner.

The quantities of these substances present in the samples from any one person may be so small that their isolation and chemical characterization is a matter of considerable difficulty. However, the small quantities of material possessing the same spectrum which occur in samples from different individuals can be pooled, and in this way sufficient material can be accumulated to permit purification and characterization by chemical methods.

The variations in the infra-red absorption spectra of such series of chromatographed fractions from individual and pooled collections of the urines of normal and diseased persons will be discussed in subsequent papers.

SUMMARY

Methods of infra-red spectrometry have been developed for the identification of the steroid metabolites found in urine. These methods can be applied both to the control of the fractionation procedures by which the individual steroid metabolites are separated and to the identification of the pure substances so obtained.

A discussion is given of the application of these methods to the estimation of the composition of binary mixtures of steroids, and to the detection of previously unrecognized steroid constituents in the sequence of components obtained by fractional chromatographic analysis.

The assistance of Mrs. M. J. Whalen of the American Cyanamid Company and of Mrs. K. Dobriner and Mrs. P. Humphries of the Sloan-Kettering Institute for Cancer Research is gratefully acknowledged by the authors.

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THE FORMATION OF CHOLESTEROL FROM *i*-CHOLESTERYL METHYL ETHER*

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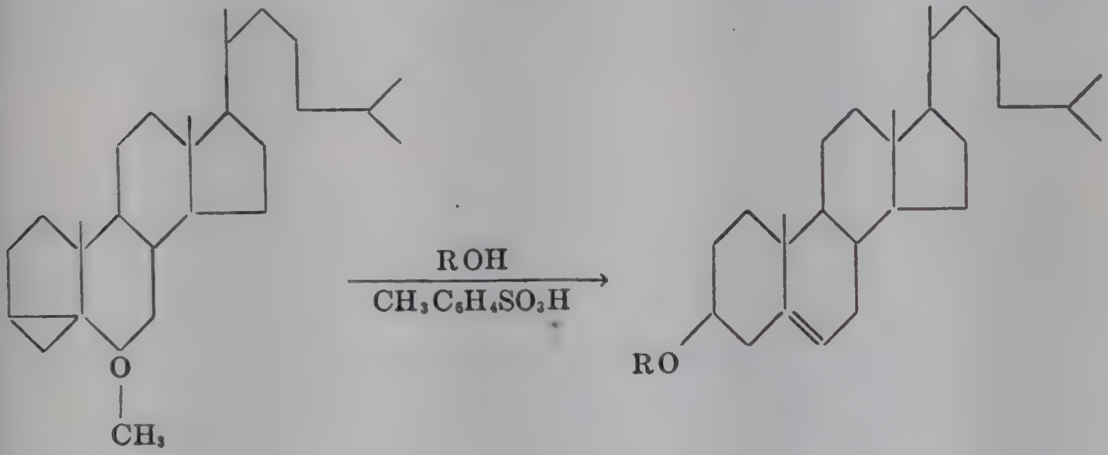
(Received for publication, September 12, 1947)

In a recent communication (1) the synthesis of cholesteryl *n*-propyl ether (IIa) from *i*-cholesteryl methyl ether (I) was reported. At that time it was stated that the reaction was of interest for the preparation of other cholesterol derivatives. The present report deals with additional applications of the above ether synthesis and the formation of cholesterol itself from *i*-cholesteryl methyl ether.



(I)

(II)

(IIa, R = C_3H_7—)

Since it has been shown (2) that the reaction of thiophenol with cholesteryl *p*-toluenesulfonate leads to the formation of a bisphenylthio compound, tentatively called 3,5-(bisphenylthio)cholestane, it would appear that in the formation of cholesteryl ethers[1] the cleavage of the carbon-oxygen bond occurs in the steroid rather than in the reacting alcohol. It was, therefore, anticipated that the methyl ether of *i*-cholesterol could be transformed to cholesteryl ethers which contain a variety of alkoxy groups.

i-Cholesteryl methyl ether was smoothly converted to *n*-propyl and *i*-propyl cholesteryl ethers by heating I and an excess of the appropriate alcohol in the presence of *p*-toluenesulfonic acid. It was then reasonable to suppose that by taking advantage of this same reaction cholesterol could b prepared. By reaction of water and I in aqueous dioxane, cholesterol wa obtained in good yield.

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] The previous reports (1, 2) discuss the formation of cholesteryl ethers from the tosyl ester (3–5) and the intermediate *i*-cholesteryl ethers, the structure of which was elucidated by Wallis and coworkers (6–8).

That the reaction shown is indeed one which involves the *i*-cholestery radical rather than simple exchanges with cholesteryl methyl ether, whic can be formed from *i*-cholesteryl methyl ether in methanol in the presenc of acid (2, 4), was clearly shown in one case investigated. Here cholestery methyl ether was heated with *n*-propanol and *p*-toluenesulfonic acid monohydrate under the same conditions which prevail in the reaction of the is compound. The normal cholesteryl methyl ether was recovered unchange in quantitative yield. It was also found that *i*-cholesteryl methyl ethe after treatment with aqueous dioxane which contained sodium acetate instead of the customary acid could be quantitatively recovered. These observations together with previous data (2, 4, 5) serve to strengthen the belief that one is dealing with a general acid-catalyzed reaction, the effect of which is the formation of 3-Δ^5-cholestenyl derivatives of anions (*viz.*, Cl^-, Br^-, I^-, HO^-, CH_3O^-, *n*-$C_3H_7O^-$, *i*-$C_3H_7O^-$, CH_3COO^-) derivable from one of the reactants.

EXPERIMENTAL

Cholesteryl n-Propyl Ether—To a solution of 300 mg. of *i*-cholesteryl methyl ether (3) in 35 cc. of *n*-propanol were added 35 cc. of a solution containing 100 mg. of *p*-toluenesulfonic acid monohydrate in *n*-propanol. The combined solutions were heated for 2 hours at 80° and then concentrated under diminished pressure to a volume of approximately 25 cc. 10 cc. of water were added slowly with stirring. The mixture was cooled, and the product was collected and washed three times with 5 cc. portions of 30 per cent aqueous *n*-propanol. The yield of cholesteryl *n*-propyl ether, m.p. 100°, was 247 mg. (77 per cent of the calculated amount). On further dilution with water the combined wash and mother liquors yielded material, m.p. 92–94°.

When the reaction was carried out as described above, except for the period of heating, which was extended to 3 hours, the yield of cholesteryl *n*-propyl ether, m.p. 100°, was 96 per cent of the calculated amount. The products, in both cases, did not depress the melting point of authentic material upon admixture.

Stability of Cholesteryl Methyl Ether to n-Propanol and p-Toluenesulfonic Acid—150 mg. of cholesteryl methyl ether were heated in 35 cc. of *n*-propanol for 2 hours in the presence of 50 mg. of *p*-toluenesulfonic acid monohydrate. The solution was concentrated under diminished pressure to a volume of approximately 20 cc. and then diluted to 100 cc. by addition of water. 150 mg. of crystals, m.p. 83.5–84°, were deposited from the cooled mixture. The quantitatively recovered cholesteryl methyl ether did not depress the melting point of an authentic sample upon admixture.

Cholesteryl i-Propyl Ether—250 mg. of *i*-cholesteryl methyl ether were treated with 70 cc. of *i*-propanol and 100 mg. of *p*-toluenesulfonic acid monohydrate at a temperature of 80° for 3 hours as described under the preparation of the *n*-propyl ether. The product weighed 255 mg. (96 per cent of the calculated amount) and melted at 132–133°. The latter is in agreement with the melting point of 132° reported by Bills and McDonald (9) for the same compound.

Cholesterol from i-Cholesteryl Methyl Ether—To a solution of 100 mg. of *i*-cholesteryl methyl ether in 50 cc. of purified *p*-dioxane were added 100 mg. of *p*-toluenesulfonic acid monohydrate in a mixture of 50 cc. of water and 50 cc. of dioxane. The combined solutions were heated for 5 hours at 80° and then allowed to cool. The solution was diluted with water to a volume of 250 cc. After the mixture had remained for several hours in the refrigerator, the crystalline product was collected and washed with water. The yield of cholesterol, m.p. 147–148°, was 92 mg. after drying *in vacuo*. This represents 95 per cent of the calculated amount. The melting point was not depressed by admixture with an authentic sample. The product, upon treatment with acetic anhydride, yielded an acetyl derivative, m.p. 114–115°, which did not depress the melting point of authentic cholesteryl acetate upon admixture.

Stability of i-Cholesteryl Methyl Ether to Aqueous Dioxane under Alkaline Conditions—A solution of 100 mg. of *i*-cholesteryl methyl ether in 50 cc. of dioxane was added to 2.00 gm. of anhydrous sodium acetate which had been dissolved in a mixture of 50 cc. of dioxane and 50 cc. of water. The combined solutions were heated at 80° for 5 hours. On dilution with water, the *i*-ether was recovered quantitatively from the mixture. The melting point of 79° was identical with that of the starting material and not depressed by admixture with the same.

SUMMARY

The conversion of *i*-cholesteryl methyl ether to cholesteryl *n*-propyl ether and to cholesteryl *i*-propyl ether by heating *i*-cholesteryl methyl ether with an excess of the appropriate alcohol and *p*-toluenesulfonic acid has been described. The reaction involved is dependent upon the marked lability of *i*-cholesteryl methyl ether, since cholesteryl methyl ether was shown to be effectually stable to *n*-propanol under conditions favorable for the reaction of *i*-cholesteryl methyl ether.

By utilizing the principles of the above reaction, which appears to be quite general, cholesterol was obtained in good yield from *i*-cholesteryl methyl ether by reaction in aqueous dioxane.

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ACCELERATION OF CATALASE ACTION

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(Received for publication, September 27, 1947)

It has been reported (1) that a concentrate prepared by alcoholic extraction of yeast cells accelerated the enzymatic decomposition of hydrogen peroxide. Because of the crude character of the preparation used, the manner in which the accelerative effect was exerted on the catalase system was unknown, but the suggestion was made that the action may have been a direct acceleration of the enzyme itself. This phenomenon has been designated as acceleration rather than activation because the accelerating substance apparently is not essential for catalase activity.

Sumner and Sisler (2) in repeating this work found that yeast extracts as prepared by them protected crude rat liver catalase from inactivation by hydrogen peroxide at 24°, but they were unable to demonstrate any acceleration by these extracts, either of the crude or of the crystalline enzyme. Protection of the enzyme by the extract had also been observed in these laboratories but this activity was not considered a unique property of the extract, since protection is also afforded by substances occurring naturally with the enzyme.

The accelerative effect of the yeast preparations on catalase action has been confirmed and extended. The further evidence obtained is presented here to show that (*a*) the catalase decomposition of hydrogen peroxide can be accelerated by the extract, (*b*) that this accelerative ability is possessed by extracts of two different strains of bakers' yeast, and (*c*) that the accelerative effect can be measured gasometrically as well as titrimetrically. Some indications as to the nature of this acceleration are also included in this paper.

EXPERIMENTAL

7 pounds of fresh Fleischmann's bakers' yeast were used for the extracts, prepared in a manner similar to that described elsewhere (3). The yeast was extracted with 2.5 liters of 95 per cent ethyl alcohol for 4 hours at a temperature of 60–70° with constant mechanical stirring, and the yeast was filtered off on a Büchner funnel. Reextraction with 50 per cent alcohol was omitted, since this did not increase the potency of the final preparation (4). The extract was then concentrated *in vacuo* at 60° to a volume of approximately 400 ml. This concentrate contained a large amount of fat and other colloidal materials which were eliminated by filtration successively through a Büchner funnel and a Seitz filter. The pH of this concentrate,

usually approximately 5.0, was adjusted to 6.8 (glass electrode) with norma sodium hydroxide. The concentrate was then sterilized by passage through a Seitz filter. Autoclaving was avoided since this caused the formation of a precipitate and a darkening of the concentrate. Dry weight determinations were made prior to adjusting the pH.

A commercial concentrate of Anheuser-Busch yeast prepared in a manner similar to that of Fleischmann's yeast was obtained from the laboratories of Sperti, Inc., Cincinnati. Before use in these experiments the commercial concentrate was filtered through a Büchner funnel, adjusted to pH 6.8 with normal sodium hydroxide, and sterilized by passage through a Seitz filter.

Decolorization of both the laboratory and the commercial types of concentrate was accomplished by treatment with norit charcoal (5). Extracts to be decolorized were diluted to 200 mg. per ml. with water and adjusted to pH 6.2. Norit charcoal (approximately 10 per cent of the total weight of the extract) was then added and the mixture heated to 60° for 10 minutes with constant stirring. The charcoal was then filtered off, the filtrate adjusted to pH 6.8, and sterilized by passage through a Seitz filter. If only a partial decolorization was effected with this procedure, the treatment was repeated.

The rate of decomposition of hydrogen peroxide by catalase in the presence and absence of yeast extracts was determined by the same permanganate titrimetric procedure as was previously used (1) and also by the gasometric method of Perlmann and Lipmann (6) with the Warburg respirometers. In the latter procedure, control manometer flasks contained 1 ml. of hydrogen peroxide (0.6 mg. of Merck's 30 per cent, diluted to 500 ml. with water), 1 ml. of 0.04 M phosphate buffer, 0.5 ml. of catalase preparation, and 0.5 ml. of distilled water.

The yeast extracts to be tested were diluted sufficiently with water so that the range of final concentrations in the manometer flask varied from 0.5 to 2 mg. per ml. This seems to be the optimum range of activity of the extract as measured either by the gasometric or the titrimetric procedure.

When the titrimetric method was employed determinations of the amount of reaction between the permanganate and the yeast extract were routinely made in the following manner. Two flasks containing 35 ml. of hydrogen peroxide and 10 ml. of buffer, were placed in the water bath. To one flask were added 5 ml. of water and to the other 4 ml. of water and 1 ml. of yeast extract containing 25 or 50 mg. per ml. of dry material (final concentration of dry material, 0.5 or 1 mg. per ml.). After a 10 minute equilibrium period 5 ml. of the solutions from each flask were removed and placed in 20 ml. of 10 per cent sulfuric acid. Each 5 ml. sample was then titrated with 0.01 N permanganate, and the difference in amount of permanganate used gave the blank correction. These corrections were then subtracted from the experimental titration values. The amount of the correction was 0.2 to 0.3 ml. of 0.01 N permanganate per 5 ml. of reaction mixture for the experiments reported here. Blanks should be determined for every batch of extract. Since the value for the blank depends upon the purity of the extract, it may in some instances be zero, as in the earlier work (1). In other cases it may be excessive and mask completely the acceleration of the enzyme.

Two types of catalase preparations were used in these experiments. One was a crude rat liver autolysate prepared as described in a previous publication (1) and the other was a purified enzyme prepared by the method of Sumner and Dounce (7). This catalase was not crystalline but had an activity of approximately 16,500 *Kat. f.* units.

Results

In Fig. 1 is shown the result of a typical experiment in which an undecolorized concentrate of the Anheuser-Busch yeast was tested for its effect on the catalytic decomposition of hydrogen peroxide by a crude rat liver enzyme as measured titrimetrically at 24°. The extract was incubated with the enzyme during the 10 minute equilibration period before addition of the hydrogen peroxide. The reaction constants of the control and experimental reactions are plotted against time (Fig. 1, Curves I and II). Extrapolating to zero time gave an 82 per cent increase of the experimental over the control reaction. This particular extract of Anheuser-Busch yeast was tested seven times at 0.5 and 1 mg. levels with qualitatively similar results. By the same method and the same conditions, similar results were obtained with three other extracts (Anheuser-Busch, decolorized and undecolorized, and Fleischmann's undecolorized). A summary of the results of these experiments is given in Table I. Reaction constants at zero time for the control systems ranged from 0.024 to 0.117. No consistent relation was observed between the percentage stimulation by an extract and the K_0 values of the control reactions. The pH of the reaction mixture, initially buffered at 6.8, was not changed with the addition of the extracts. Moreover, the reaction mixture in both the control and experimental flasks did not change in hydrogen ion concentration during the course of the reaction.

The values given in both Table I and Fig. 1 (Curve II) were calculated after correction for the interaction of permanganate and yeast extract. It was not necessary, however, to make such corrections to show the acceleration by these extracts, as is evident from the uncorrected data presented in Curve III, Fig. 1.

The extracts themselves in the absence of the catalase did not decompose the hydrogen peroxide under the conditions of the experiments, confirming early observations (1) and indicating that the acceleration of the enzymatic decomposition was not due to a catalyst in the extract.

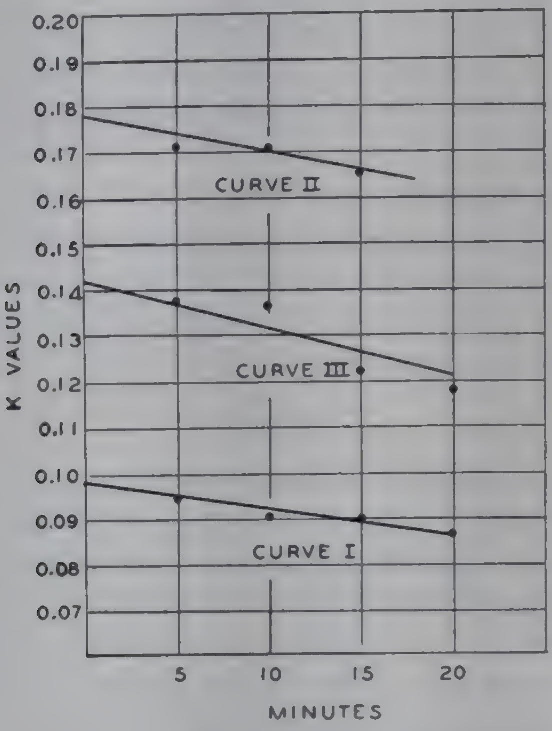

FIG. 1. Effect of yeast extract on decomposition of hydrogen peroxide by a crude catalase. Temperature 24°. Curve I, control (hydrogen peroxide, buffer, enzyme); Curves II and III, control + yeast extract (1.0 mg. per ml., final concentration). Curve II, corrected data; Curve III, uncorrected data.

TABLE I

Acceleration of Catalase Activity by Different Yeast Extracts As Measured Titrimetrically with Crude Catalase at 24°

Range of K_0 values of control systems, 0.024 to 0.117.

Type of yeast extract	Concentration	No. of times tested	Range of activity	Average increase
	mg. per ml.		*per cent*	*per cent*
Anheuser-Busch, No. 134-5	0.5	2	42–47	45
	1.0	5	24–80	47
“ “ 154-5	1.0	3	68–77	72
	2.0	1		71
“ “ 154-5, decolorized	1.0	2	65–89	77
Fleischmann, No. A	1.0	5	32–93	71

To determine to what extent this acceleration of catalase decomposition of hydrogen peroxide is due to a protection of the enzyme against deterioration by the hydrogen peroxide, several experiments were performed in which a crude rat liver catalase was allowed to decompose the hydrogen peroxide at 0°. The reaction constants of the decomposition remained the same during a 20 minute experimental period, indicating no deterioration of the enzyme (Table II). The addition of a yeast extract (1 mg. per ml., final concentration of Fleischmann's undecolorized concentrate) resulted in an increase in the reaction constant over the control at the first period of measurement, with no change in this reaction rate during the remainder of the experimental period (Table II). The same results were obtained with a more active enzyme ($K_0 = 0.076$).

When the extracts were not allowed to stand with the enzymes during the equilibration period but were added simultaneously with the hydrogen peroxide to the enzyme-buffer mixture, the reaction constants of the experimental solutions increased during the course of the decomposition of the hydrogen peroxide. Generally the maximum reaction constant was reached at 5 minutes from the time of the addition of the hydrogen peroxide. The same phenomenon was observed when the extract was tested at 0° in systems containing the purified enzyme, *Kat. f.* 16,500 (Fig. 2). The reaction constants of the experimental solutions containing the extracts increased generally for the first 15 minutes after addition of the hydrogen peroxide. Similar results were obtained in five experiments with the same extract (Fleischmann's Extract A). Allowing the extract to stand with the enzymes at 0° for various periods of equilibration did not change the course of reaction or the rise in the reaction constants. The acceleration of catalase decomposition of hydrogen peroxide by a yeast extract was confirmed independently by Sister M. Julitta Bomkamp of Villa Madonna College, who used both a crude and a crystalline (*Kat. f.* 25,300) enzyme (personal communication).

TABLE II

Effect of Yeast Extract (Fleischmann) on Crude Catalase at 0°

The experimental values are the average of two experiments.

Flask	Yeast extract concentration	K_0 values					Increase
		5 min.	10 min.	15 min.	20 min.	0 min., extrapolated	
	mg. per ml.						*per cent*
Control	0.0	0.025	0.023	0.023	0.023	0.024	
Experimental	1.0	0.0425	0.0400	0.0455	0.0420	0.0422	78

To eliminate the difficulties inherent in the use of the titrimetric procedure, in which there is a reaction between permanganate and yeast extract, necessitating blank determinations and correction factors, the effect of the yeast extract on the enzymatic decomposition of hydroge peroxide was measured by the gasometric technique of Perlmann an Lipmann (6). The course of the reaction was followed by the volumetri determination of the oxygen evolved. The extract in final concentration of 1 and 2 mg. per ml. increased the evolution of oxygen (Table III). In twenty similar experiments with two yeast extracts (crude extracts of Fleischmann and Anheuser-Busch yeast) qualitatively similar results were obtained. In the absence of the enzyme the extracts in the same concentra-

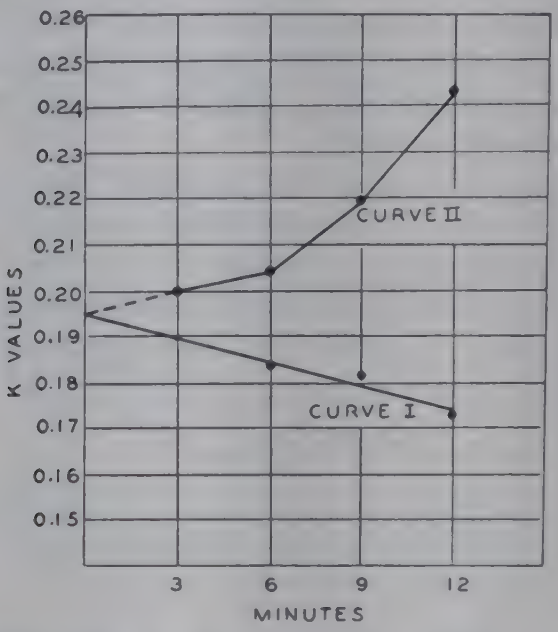

FIG. 2. Effect of yeast extract on decomposition of hydrogen peroxide by a purified enzyme (*Kat. f.* 16,500). Curve I, control (hydrogen peroxide, buffer, enzyme); Curve II, control + extract (1.0 mg. per ml., final concentration).

TABLE III

Effect of Yeast Extract on Catalase As Determined by Gasometric Procedure at 30°

The results are the average of two experiments. Twenty other similar experiments confirm these observations.

Time	O_2 evolved		
	Control*	Yeast extract, 2.0 mg. per ml.	Yeast extract, 1.0 mg. per ml.
min.	*c.mm.*	*c.mm.*	*c.mm.*
5	23.30	33.70	32.20
10	47.23	59.93	57.74
15	59.92	72.85	70.88
20	71.40	88.02	85.75

* Control = hydrogen peroxide, buffer, and crude enzyme.

tions did not decompose the hydrogen peroxide. It is apparent that the oxidizable materials of the extract did not react appreciably with the oxygen liberated during the course of the decomposition of the hydrogen peroxide.

DISCUSSION

With the permanganate titration procedure it was found that the permanganate reacted to a slight degree (0.2 to 0.3 ml. of 0.01 N permanganate per 5 ml. of reaction mixture) with substances in the yeast extracts, presumably oxidizable materials. The question immediately arose as to whether a reaction occurred between the materials in the extract and the hydrogen peroxide in the presence of the catalase, a possible cyclic process similar to that described by Keilin and Hartree (8). That such is not the case is shown by the experiment (Table IV) in which the difference in the amount of permanganate required at zero time in the titration of 5 ml. of the reaction mixtures in the presence and absence of yeast extract (blank determination) was the same as that after complete decomposition of the hydrogen peroxide (1 hour).

TABLE IV

Reaction of Yeast Extract (Fleischmann's, Undecolorized) with Permanganate before and after Decomposition of Hydrogen Peroxide by Catalase

Values given in ml. of 0.01 N potassium permanganate.

Experiment No.	0 time, enzyme absent			60 min., enzyme present		
	Yeast extract absent	Yeast extract present, 1 ml.	Difference	Yeast extract absent	Yeast extract present, 1 ml.	Difference
1	4.69	4.85	0.24	0.09	0.52	0.43
2	4.69	4.85	0.24	0.08	0.32	0.24
3	4.72	5.13	0.41	0.08	0.33	0.25
Average..	4.70	4.94	0.29	0.08	0.39	0.31

Since the reaction between yeast extract and permanganate lowers the values for acceleration of the enzyme, the blank correction should be subtracted from the titration values for the experimental reaction mixture. In Fig. 1, Curves II and III respectively demonstrate the corrected and uncorrected values for the rate of reaction, and the amount of acceleration is significant in the uncorrected Curve III.

By the use of the gasometric procedure, acceleration of the enzymatic decomposition of hydrogen peroxide can be demonstrated without the complications inherent in the titrimetric procedure. The increased oxygen production obtained in the presence of the yeast extract indicates that there is no reaction between the hydrogen peroxide and the extract in the presenc of the catalase.

SUMMARY

By means of the permanganate titration procedure, alcoholic extracts o two strains of bakers' yeast were found to increase the rate of enzymati decomposition of hydrogen peroxide. Systems containing both crude anc purified catalase were found to be accelerated by the extracts. The extrac was partially purified, with concentration of its activity, by treatment witl norit charcoal at pH 6.2. The gasometric method of Perlmann anc Lipmann (6) was more satisfactory for demonstration of the acceleration o catalase by the extracts, since it eliminated the necessity for the blank corrections required because of the reaction between permanganate anc the extract in the titrimetric procedure. The acceleration of hydroger peroxide decomposition was shown by the acceleration of oxygen evolution

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16-SUBSTITUTED STEROIDS

IV. 16-KETO-α-ESTRADIOL AND 16-KETOESTRONE

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In 1942 one of us (1) submitted 16-oximinoestrone to reduction with zinc and aqueous acetic acid and obtained an α-ketol which was at first supposed to be one of the two theoretically possible 16-hydroxyestrones. Subsequent work (2), however, indicates beyond reasonable doubt that a ketol which results on the zinc-acetic acid reduction of a 16-oximino-17-ketosteroid (Stodola reduction[1] (3)) is actually a 16-keto-17(α)-hydroxysteroid. Detailed structural considerations are reserved for subsequent treatment, it being the purpose here only to describe preparation and characterization.

Because of the interest (4, 1, 5) in the possibility that 16-ketoestrone and 16-keto-α-estradiol may be intermediates along the normal (or abnormal) metabolic pathway of the estrogenic hormones, it seemed of importance to present a careful report on the syntheses of these two steroids in order that they might become accessible to all interested investigators.

16-Keto-α-estradiol (IIIa) results on the zinc-acetic acid reduction of either 16-oximinoestrone (IIa) or of 16-ketoestrone (IV). This α-ketol forms, in nearly quantitative amount, a characteristic monooxime. On treatment with benzoyl chloride in the Schotten-Baumann method it yields a 3-monobenzoate. On treatment with benzyl chloride in the Claisen procedure there is obtained a 3-benzyl ether[2] (IIIb) which is identical with the α-ketol furnished by the Stodola reduction of 16-oximinoestrone 3-benzyl ether (IIb). In our hands 16-keto-α-estradiol yielded no precipitate with digitonin.

By following closely the method (6) recently described for the preparation of 16-ketoestrone 3-methyl ether it is possible to obtain, from 16-

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* A portion of the research presented in this paper was performed by one of us while a National Research Council Fellow in the Medical Sciences (1941–42) at the Department of Biochemistry, College of Physicians and Surgeons, Columbia University. Another portion was done at the United States Standard Products Company, Woodworth, Wisconsin.

[1] The term Stodola reduction is here applied to the reduction of any 16-oximino-17-ketosteroid with zinc and acetic acid. In this reaction, originally applied by Stodola *et al.* (3), there is curiously produced no amine, but an α-ketol instead.

[2] The methyl ether melting at 174–177° and its oxime melting at 175–177°, as originally cited (1), are not now believed to represent homogeneous substances.

oximinoestrone, 16-ketoestrone (IV) with the phenolic hydroxyl unsubstituted. We found, however, that the free phenol is much more soluble in the acid reaction mixture, thus necessitating the introduction of a salting-out procedure to obtain the dione in crystalline form. 16-Ketoestrone was characterized as the dimethoxime and the dioxime. Upon reduction with zinc-acetic acid, yellow 16-ketoestrone yields white 16-keto-α-estradiol (IIIa) which in turn gives only a monooxime upon treatment with excess hydroxylamine acetate. The dioximes formed by the oximation of 16-oximinoestrone and by the direct oximation of 16-ketoestrone are identical. Both dioximes furnish a greenish yellow-colored complex with cupric ion. This color reaction may be of possible utility in the detection of 16-ketoestrone in tissue or fluid, should it be present as postulated by Marrian (4).

Estrone is transformed, in 93 per cent yield, to estrone 3-benzoate of sufficient purity for subsequent nitrosation (7). Such estrone benzoate yields 80 per cent crude 16-oximinoestrone (7) which may be purified with 20 per cent loss. 16-Keto-α-estradiol of acceptable quality may ordinarily be obtained from pure 16-oximinoestrone in a yield of 75 per cent. From pure 16-oximinoestrone the yellow dione, 16-ketoestrone, is procured in yields of 60 per cent of the theoretical amount. Therefore, in these partial syntheses from estrone, the commonly obtainable over-all yields of 16-keto-α-estradiol and of 16-ketoestrone are 45 and 36 per cent respectively. The highest yield of crude 16-oximinoestrone from estrone benzoate ever observed was 96 per cent (7), and good quality 16-keto-α-estradiol was once obtained from pure 16-oximinoestrone in 90 per cent yield. Recalculated on the basis of these unusual yields, the over-all percentages for 16-keto-α-estradiol and 16-ketoestrone would rise to 63 and 42 respectively. These latter figures are of interest in serving to indicate possible maxima.

EXPERIMENTAL[3]

Reduction of 16-Oximinoestrone to 16-Keto-α-estradiol—Pure 16-oximinoestrone (IIa) (0.61 gm.) was covered with 20 cc. of water, and to the mixture 1.6 gm. of zinc dust plus 20 cc. of acetic acid were added. The mixture was swirled over a free flame until solution of the steroid had become complete; the solution was then refluxed over the zinc for a period of 1 hour, allowed to cool somewhat, and decanted. The zinc was twice rinsed with 3 cc. portions of acetic acid and the combined acetic acid solutions diluted with several volumes of water. After a day's standing at room temperature the resulting white crystals were filtered, washed with water, and dried in the oven (yield, 0.55 gm.). A subsequent recrystallization from 60 cc. of 50 per cent acetic acid (ice box) gave 0.44 gm. of α-ketol (IIIa) melting at

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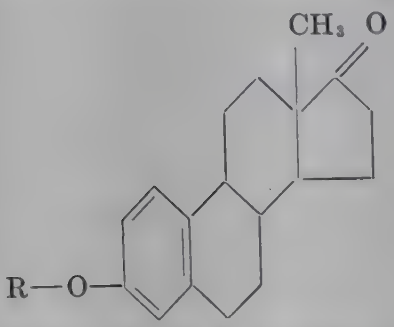
[3] All melting points reported are uncorrected.

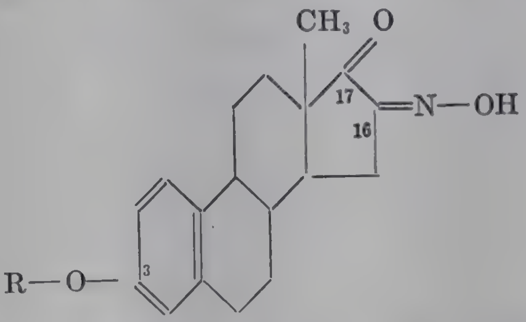
232.5–234° (turning orange in color at the melting point[4]). Another recrystallization from 50 per cent acetic acid gave white plates in fan-shaped clusters which melted at 234–237° to an orange liquid. This melting point was not influenced by further crystallization from aqueous acetic acid.

Analysis—$C_{18}H_{22}O_3$. Calculated. C 75.49, H 7.75
Found. " 75.48, " 7.73
$[\alpha]_D^{29.5} = -102°$ (27.24 mg. in 5.00 cc. of ethanol)

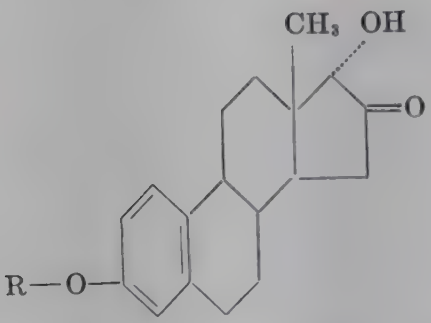
We once obtained from 0.50 gm. of pure 16-oximinoestrone 0.43 gm. of 16-keto-α-estradiol melting at 231–233.5° with color change. The yield in this reaction is usually, however, in the neighborhood of 75 per cent of the theoretical amount.



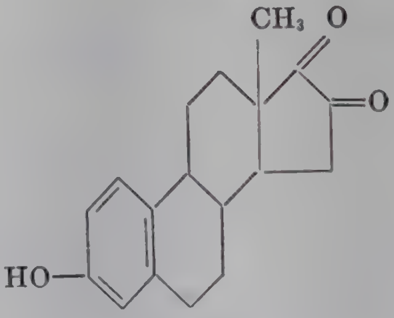
Ia, R = H; Ib, R = $CH_2—C_6H_5$



IIa, R = H; IIb, R = $CH_2—C_6H_5$



IIIa, R = H; IIIb, R = $CH_2—C_6H_5$



(IV)

Attempted Digitonide Formation with 16-Keto-α-estradiol—To 18.6 mg. of pure 16-keto-α-estradiol were added 1.0 cc. of 80 per cent ethanol and 8.0 cc. of a saturated solution of digitonin in 80 per cent ethanol. The mixture was warmed gently to effect solution of the steroid and then stoppered and allowed to remain at room temperature. No precipitation had occurred in this solution after a period of 14 days.

16-Keto-α-estradiol Oxime—A solution of 45 mg. of pure 16-keto-α-estradiol (IIIa), 50 mg. of hydroxylamine hydrochloride, and 100 mg. of crystalline sodium acetate in 4.5 cc. of ethanol plus 0.5 cc. of water was refluxed gently on the steam bath for 2 hours. Water (10 cc.) was then added, the reflux condenser removed, and boiling continued until crystallization had commenced. Crystallization was allowed to continue in the ice box. The yield of oxime was 45 mg. (m.p. 220–221°, with decomposition). After treatment with charcoal and recrystallization from aqueous methanol the melting point rose to 224.5–225°, with decomposition (yield, 32 mg.).

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[4] All 16-keto-17(α)-hydroxysteroids so far examined which melt in the neighborhood of 200° or more have been observed to turn a yellow or orange color at the melting point.

Analysis—$C_{18}H_{23}O_3N$. Calculated, N 4.65; found, N 4.61

16-Keto-α-estradiol 3-Monobenzoate—16-Keto-α-estradiol (44 mg.) was dissolved rapidly in 30 cc. of cold 0.5 N sodium hydroxide and 0.5 cc. redistilled benzoyl chloride was added at once. The stoppered mixture was shaken well for 15 minutes and allowed to remain at room temperature overnight. The precipitated benzoate was then filtered, washed with dilute sodium hydroxide, washed with water, and then dried in the desiccator. After crystallization from 25 cc. of methanol (plus 3 drops of acetic acid) 30 mg. of crystals melting at 234.5–236° (turning orange in color) were obtained. Treatment with charcoal and recrystallization from acetone yielded 16 mg. of monobenzoate melting at 241.5–243.5° (orange color).

Analysis—$C_{25}H_{26}O_4$. Calculated. C 76.90, H 6.71
Found. " 76.57, " 6.83

Nitrosation of Estrone 3-Benzyl Ether—Estrone 3-benzyl ether was nitrosated by the method (8) previously described for the 3-methyl ether. The yield of crude 16-oximinosteroid (m.p. 163–165°, with decomposition) was 70 per cent. Subsequent recrystallization, once from methanol, twice from chloroform-ethanol, and once from ethanol with the use of charcoal, yielded fine, very pale yellow needles which decomposed at 193.5–195.5° (IIb).

Analysis—$C_{25}H_{27}O_3N$. Calculated. N 3.60
Found. (*a*) " 3.51
(*b*) " 3.55

16-Keto-α-estradiol 3-Benzyl Ether—To 395 mg. of crude 16-oximinoestrone 3-benzyl ether (IIb) and 13 cc. of water 1.5 gm. of zinc dust and 26 cc. of acetic acid were added. The mixture was refluxed for 80 minutes, and the supernatant fluid at once decanted from the zinc, the latter being washed twice with 6 cc. portions of acetic acid. To the combined acetic acid solution 25 cc. of water were added and the crystallization allowed to take place during 24 hours. Tiny white needles (352 mg.) were obtained. These melted at 192–195° (yellow color). Another crystallization from queous acetic acid raised the melting point to 196–198.5° (yellow color). reatment with charcoal and recrystallization from aqueous acetone did ot improve this melting point[5] (IIIb).

Analysis—$C_{25}H_{28}O_3$. Calculated. C 79.75, H 7.50
Found. (*a*) " 79.72, " 7.47
(*b*) " 79.62, " 7.40

Benzylation of 16-Keto-α-estradiol—A solution of 399 mg. of pure 16-keto-α-estradiol and 0.80 cc. of benzyl chloride in 20 cc. of ethanol was refluxed over 0.90 gm. of anhydrous potassium carbonate (steam bath). Potassium chloride soon commenced to precipitate. After a total of 1.5 hours refluxing, water (5 cc.) was then added to the reaction mixture and the latter allowed to stand overnight in the ice box. Fine, white crystals (473 mg.) were obtained. On recrystallization from 50 cc. of acetic acid plus 15 cc. of water, 416 mg. of tiny white needles melting at 195–197° (yellow color) were obtained. These gave no melting point depression with the 16-keto-α-estradiol 3-benzyl ether (IIIb) obtained in the preceding section.

Acetylation of 16-Keto-α-estradiol 3-Benzyl Ether—To 0.37 gm. of pure 16-keto-α-estradiol 3-benzyl ether dissolved in 5 cc. of dry pyridine, 3 cc. of acetic anhydride were added. The two phases were mixed well and allowed to remain 24 hours at room temperature with occasional swirling. The steroid acetate was then precipitated by the aid of 200 cc. of ice-cold water. After several hours in the ice box, the acetate was filtered and washed copiously with water. A recrystallization from aqueous acetone-ethanol gave 0.38 gm. of felt-like needles melting at 141°.

Analysis—$C_{27}H_{30}O_4$. Calculated. C 77.48, H 7.23
Found. (*a*) " 77.50, " 7.23
(*b*) " 77.57, " 7.16

16-Ketoestrone—To 500 mg. of pure 16-oximinoestrone (IIa) and 5.0 gm. of anhydrous sodium sulfite, 20 cc. of acetic acid were added. This mixture was heated on the steam bath for 15 minutes with frequent stirring, and then diluted with 5 cc. of water. Heating was continued for 45 minutes longer with almost continuous stirring. After having been cooled to room temperature the mixture was transferred to a separatory funnel with the aid of 200 cc. of 3 per cent sodium bisulfite. The clear aqueous phase was shaken with 250 cc. of peroxide-free ethyl ether and separated. The ether phase was extracted again with 50 cc. of 3 per cent sodium bisulfite. To the combined bisulfite phases 30 cc. of concentrated hydrochloric acid and a few small chips of porous plate were added. The acidified solution was heated on the steam bath with almost continuous swirling until ebullition from the porous stones had ceased. Immediately 50 gm. of pure sodium chloride (fine crystals) were added and heating continued for 15 minutes longer with frequent swirling. The mixture was allowed to cool to room temperature and remain so overnight. After filtration, copious washing with water, and drying in the desiccator, 284 mg. of microscopic yellow crystals were obtained. These sintered at 223° and melted, with decomposition, at 231–235°. Of this material, 100 mg. were made into the bisulfite addition compound and then hydrolyzed to give 66 mg. of pale yellow crystals (microscopic) decomposing at 234–238° (IV).

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[5] Several samples of 16-keto-α-estradiol 3-benzyl ether melting at 200° have been encountered. It is possible that the rapidity of temperature rise in the melting point bath is here a factor.

Analysis—$C_{18}H_{20}O_3$.		
	Calculated.	C 76.03, H 7.09
	Found. (*a*)	" 75.39, " 7.13
	(*b*)	" 75.34, " 7.09

Reduction of 16-Ketoestrone to 16-Keto-α-estradiol—400 mg. of 16-ketoestrone (m.p. 233–236°, with decomposition) (IV) were covered with 40 cc. of 50 per cent acetic acid and a small spatula edge of zinc dust was added. The mixture was heated on the steam bath with swirling, and more zinc added as the yellow color of dissolved dione appeared. After the steroid had been totally dissolved, zinc dust was added until the solution could not be further decolorized. The supernatant fluid was decanted from the zinc, the latter washed twice with small portions of acetic acid, and the combined acid portions diluted with 8 volumes of water. After having been allowed to crystallize overnight (room temperature), the white crystals were filtered and washed well with water. The yield, after drying, was 360 mg. The crude α-ketol was recrystallized from aqueous acetic acid to give 288 mg. of material melting at 233.5–235.5° (orange color) (IIIa). This melting point was not depressed after admixture with 16-keto-α-estradiol obtained by the Stodola reduction.

Oximation of this α-ketol (40 mg. of ketol, 60 mg. of hydroxylamine hydrochloride, 120 mg. of crystalline sodium acetate) gave 32 mg. of oxime decomposing at 221–222°.

16-Ketoestrone Dimethoxime—49 mg. of 16-ketoestrone (m.p. 231–235°, with decomposition) were covered with 2.00 cc. of ethanol containing 75 mg. of methoxylamine hydrochloride and then 0.50 cc. of aqueous sodium acetate (≈ 134 mg. of $NaOAc \cdot 3H_2O$) was added. This mixture was diluted with 3 cc. of ethanol and left 1 hour at room temperature with frequent swirling. The solution was then refluxed for 1 hour, diluted with 5 cc. of water, and distilled to turbidity. After 2 days in the ice box, the dimethoxime was filtered and recrystallized from aqueous methanol; yield, 52 mg. melting at 197.5–198.5°. Recrystallization from absolute methanol yielded 35 mg. of flat needles with only a faint touch of yellow color; these melted at 204–205° with decomposition.

Analysis—$C_{20}H_{26}O_3N_2$. Calculated. N 8.18
Found. (*a*) " 8.12
(*b*) " 8.01

Oximation of 16-Oximinoestrone—To a mixture of 46 mg. of pure 16-oximinoestrone, 100 mg. of hydroxylamine hydrochloride, and 200 mg. of crystalline sodium acetate were added 5.0 cc. of ethanol and 1.0 cc. of water. The mixture was refluxed 1 hour, treated with charcoal, diluted with 20 cc. of water, and heated on the steam bath until alcohol ceased to distil over. The crystalline dioxime, after having been allowed to stand for several hours at room temperature, was filtered, washed well with water, and dried in the desiccator. The dried dioxime weighed 42 mg. and decomposed at 217–218°.

Analysis—$C_{18}H_{22}O_3N_2$. Calculated, N 8.91; found, N 8.65

The most characteristic property of this dioxime is the colored complex which it gives with cupric ion. No colored complex is obtained with nickelous or cobaltous ion. To obtain the color reaction the dioxime is first dissolved in a small amount of alcohol and the alcoholic solution diluted with a very weak aqueous solution of cupric acetate. A yellowish green color formation results, and this color is much more intense than the original very faint blue of the copper.

Occasionally a higher melting form of 16-ketoestrone dioxime is encountered. We have not been able to obtain it with consistency and do not know the exact procedure to employ to procure it. This crystalline modification melts at 230–231° with decomposition. It gives the same color reaction with cupric ion, and is therefore considered to be a crystalline modification rather than a geometric isomer of the dioxime decomposing at 217–218°.

Analysis—$C_{18}H_{22}O_3N_2$. Calculated, N 8.91; found, N 8.73

Oximation of 16-Ketoestrone—A solution of 66 mg. of hydroxylamine hydrochloride in 5.0 cc. of ethanol was added to 40.7 mg. of 16-ketoestrone (m.p. 231–235°, with decomposition), and at once 1.0 cc. of aqueous sodium acetate ($\approx$ 66 mg. of $NaOAc \cdot 3H_2O$) was mixed in. Solution was rapidly effected, and the reaction mixture was then left 4 hours at room temperature. The solution was next refluxed mildly for 1.5 hours, diluted with 5 cc. of water and ethanol was distilled off until crystallization in the hot solution had commenced. The white crystals, filtered after a day at room temperature, were treated with charcoal and recrystallized once from aqueous ethanol and once from aqueous propanol. The yield was 39 mg., melting at 218–219° with decomposition. Two subsequent recrystallizations from aqueous methanol did not raise this melting point.

Analysis—$C_{18}H_{22}O_3N_2$. Calculated. N 8.91
Found. (*a*) " 8.84
(*b*) " 8.73

This dioxime gave the color reaction with cupric ion as described in the preceding section. It showed no depression of melting point after admixture with the dioxime (lower melting form) prepared by oximation of 16-oximinoestrone.

SUMMARY

The partial syntheses of 16-keto-α-estradiol and of 16-ketoestrone are described in detail. The characterization of these two substances is described.

From estrone, 16-keto-α-estradiol may be prepared in 45 per cent and 16-ketoestrone in 36 per cent yields.

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The senior author desires especially to extend his appreciation to Professor Hans T. Clarke, College of Physicians and Surgeons, Columbia University, New York, under whose counsel this research was first commenced.

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METABOLISM OF SODIUM SELENATE AND SELENITE BY THE TISSUES*

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Various workers investigating the toxic action of selenium postulated that changes in the forms of selenium occur in the animal. The mechanism whereby the selenium is changed by the tissues has been the subject of speculation, but no experimental evidence has been reported up to the present time.

Hofmeister (1) has reported that the salts of selenium and tellurium are reduced to metallic form in the body; afterwards methyl compounds are formed and these volatile compounds are eliminated by the lung. Challenger (2) pointed out that "this conclusion lacks experimental verification." Schultz and Lewis (3) studied the elimination of volatile selenium by the respiratory system when sodium selenite was injected subcutaneously. They reported that within 8 hours from 17 to 52 per cent of the injected selenium was eliminated as a volatile compound which could be absorbed by concentrated sulfuric acid. The respiratory excretion of the volatile compounds was not increased by increasing the potential source of methyl groups by methionine or choline chloride. McConnell (4), in time-excretion studies of exhaled selenium after the subcutaneous injection of radioactive selenium in the form of selenate, found that 3 to 10 per cent of the original dose was exhaled during the first 6 hours. Potter (5) investigating the action of various compounds, among them sodium selenate and selenite, on yeast respiration in a glucose substrate observed that with selenite the inhibition began at once, while with selenate the oxygen uptake was reduced only at the end of 2 hours. Potter and Elvehjem (6) studying the succinoxidase as it occurs in chick tissues found that the enzyme system was inhibited by cyanide, sodium selenite, and sodium arsenite, while sodium fluoride, sodium selenate, and arsenate were relatively non-toxic at the end of 1 hour. They suggested that the selenate must be reduced to selenite by tissues before any toxicity results.

A survey of the literature reveals no experimental evidence on the site of conversion of selenate to selenite, formation of volatile selenium, or reduction to elemental selenium. These changes have been postulated, but up to the present time no experiments have been made with organs taking part in these transformations.

In our studies in investigating the physiological action of selenate and selenite on isolated guinea pig intestine, we observed that 75 parts pe million of selenate did not interfere with the normal function of this tissue while 8 parts per million of selenite completely inhibited the intestina motility. These experiments indicated that selenate was an atoxic form at least to the intestine, and that selenite was the toxic form of selenium which could inhibit the normal function of the intestine. If the selenate is converted into selenite before it can exert its toxic effect the site of conversion is not isolated intestine. Details of these experiments will be published in another report. In order to get some evidence on the metabolism of sodium selenate and selenite, we attempted to study the effect of various fresh tissues and autoclaved liver tissue on these compounds. In this report we present the results of these experiments.

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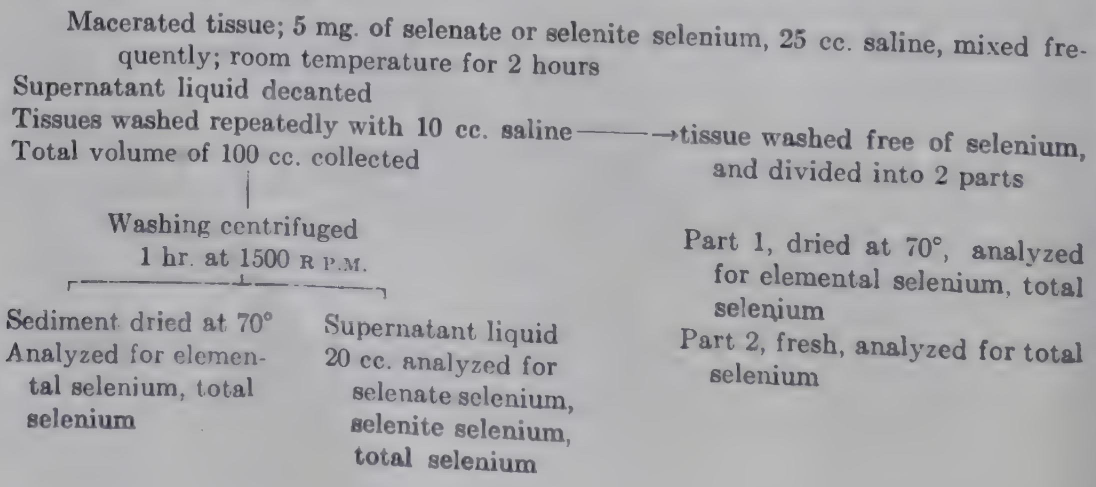
* Approved for publication by the Director of the Experiment Station.

EXPERIMENTAL

Fresh beef liver, spleen, and blood were used in the experiments, since these organs in animal poisoning have a high concentration of selenium (7). Each experiment was repeated three times, and the results given are the closest agreement of two experiments. The tissues were used 30 minutes after the animals were slaughtered. If the tissues were kept longer, there was considerable variation in their action on selenate and selenite. Tissues of other animals, such as sheep, guinea pigs, and rats, showed considerable difference in their action on selenate and selenite. It is known that different species of animals show variations in susceptibility to the toxic action of selenium. In order to investigate the observed differences in the physiological action of selenate and selenite on guinea pig intestine, we have included this organ also in our study. We have observed some variations in the results by changing the temperature, but our report deals only with the results obtained at room temperature.

In each experiment we used 20 gm. of tissues and 5 cc. of saline solution containing 5 mg. of sodium selenate and selenite selenium.

The method of procedure and scheme of fractionation are presented in the accompanying diagram.



Macerated tissue; 5 mg. of selenate or selenite selenium, 25 cc. saline, mixed frequently; room temperature for 2 hours

Supernatant liquid decanted

Tissues washed repeatedly with 10 cc. saline → tissue washed free of selenium, and divided into 2 parts

Total volume of 100 cc. collected

Washing centrifuged 1 hr. at 1500 R.P.M.

Sediment dried at 70° Analyzed for elemental selenium, total selenium

Supernatant liquid 20 cc. analyzed for selenate selenium, selenite selenium, total selenium

Part 1, dried at 70°, analyzed for elemental selenium, total selenium

Part 2, fresh, analyzed for total selenium

The first results with the liver indicated that there was a considerable oss of selenium when selenite salt was in contact with the tissues and less with selenate. Since each fraction of the tissue and supernatant liquid was analyzed, we concluded that this loss was due to volatile selenium. Therefore, we inserted several absorption flasks, using procedures similar to that of McConnell (4) or Schultz and Lewis (3) but with some variation n the absorbents used. We used the following absorbents: hydrobromic acid followed by water, sulfuric and nitric acids and sodium hydroxide, or only sulfuric and nitric acids. These modifications were instituted when we became aware that the recovery of volatile selenium was not quantitative.

Analysis for selenate, selenite, and elemental selenium was carried out according to Byers *et al.* (8). Determinations of total selenium and of volatile selenium, determined as total selenium, were carried out according to the method of Kline (9).

Results

Effects of Liver Tissue on Selenate and Selenite—Changes in the form of selenium occurred when sodium selenate and selenite were in contact with fresh liver tissue. Table I gives the results obtained. 26 per cent of the original selenate was converted to selenite, indicating that the liver is a site where reduction from selenate to selenite takes place. Calculated on the basis of the selenium added, 17 per cent of selenate and 31 per cent of selenite were changed to volatile selenium. In the selenate experiments we have recovered an average of 5 per cent and in the selenite an average of 15 per cent of volatile selenium. In experiments *in vivo*, with the same absorbents, other workers were able to recover larger amounts of volatile selenium. The only explanation we have for the lower values in our experiments is that the forms of volatile selenium *in vitro* may be different from those formed *in vivo*.

Selenite was reduced to elemental selenium by the liver; part of this was present in the centrifuged sediment and part in the tissues. No reduction of selenate to elemental selenium took place under our present experimental conditions with liver.

In addition to the reduction of selenate to selenite, and volatile selenium, in the supernatant liquid, "combined selenium" was present, as can be seen from the results. Evidence for a "combined selenium" is the fact that the total selenium content of the supernatant liquid was always greater than the sum of the selenate and selenite selenium contained therein. This difference in selenium values is called "combined selenium" in our tables. Whether this selenium was in colloidal or organic form combined with the water-soluble proteins, we cannot state at the present time.

In order to investigate the factors responsible for the changes in the forms of selenium by the fresh tissues, we have studied the effect of autoclaved liver on selenite and selenate. The results of the experiments, given in Table II, indicate that only fresh tissue has the property of decomposing the selenate to selenite and converting the selenite into elemental and volatile selenium. We have found that selenite has a greater affinity for tissues than selenate whether it is in contact with fresh or autoclaved tissue. This affinity of selenite for the tissues may account for its instability, rapid volatilization, and reduction to elemental selenium when it is in contact with fresh tissue. The factor or factors which accomplish the decomposition of

TABLE I

Chemical Decomposition of Sodium Selenate and Selenite in Contact with Fresh Beef Liver for 2 Hours at Room Temperature

Form of Se added	Se recovered from	Form of Se recovered from various fractions				Combined Se*	Volatile Se recovered
		Selenite	Selenate	Elemental	Total		
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Sodium selenate	Supernatant liquid	26.0	23.4	0	49.8	0.4	
	Centrifuged sediment from supernatant liquid			0	3.6		
	Macerated liver			0	28.9		5.0
Sodium selenite	Supernatant liquid	4.0			33.7	29.7	
	Centrifuged sediment from supernatant liquid			3.6	9.7		
	Macerated liver			4.9	26.3		15.0

* Combined selenium = total Se − (selenate + selenite).

TABLE II

Effect of Autoclaved Liver on Sodium Selenate and Selenite

Form of Se added	Se recovered from	Form of Se recovered* from various fractions		
		Selenite	Selenate	Total
		per cent	*per cent*	*per cent*
Sodium selenate	Supernatant liquid	0	85.7	85.0
	Centrifuged sediment from supernatant liquid			11.5
	Macerated liver			2.5
" selenite	Supernatant liquid	20	0	20.2
	Centrifuged sediment from the supernatant liquid			33.6
	Macerated liver			46.3

* Elemental, none.

selenate and selenite are heat-labile, since the forms of selenium added were not altered by the autoclaved tissue. Perhaps the factors responsible for the changes are enzymatic in nature.

Effect of Blood on Sodium Selenate and Selenite—Since blood contains large amounts of selenium during selenium poisoning, we studied the effect of freshly drawn beef blood on selenite and selenate selenium. The method of procedure was the same as that outlined previously.

TABLE III

Chemical Changes in Sodium Selenate and Selenite in Contact with Beef Blood at Room Temperature for 2 Hours

Form of Se added	Tissue used	Se recovered from	Form of Se recovered from various fractions				Combined Se*	Volatile Se recovered
			Selenite	Selenate	Elemental	Total		
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Sodium selenate	Whole blood	Supernatant liquid	6.3	66.0	0	95.3	23.0	
		Centrifuged red blood cells			0	3.2		0.6
	Red blood cells	Supernatant liquid	0	80.8		91.3	10.5	
		Red blood cells			0	8.2		
	Plasma	Supernatant liquid	4.8	36.6		97.0	55.6	2.4
Sodium selenite	Whole blood	Supernatant liquid	9.1			84.1	75.0	1.2
		Red blood cells			0.9	14.7		
	Red blood cells	Supernatant liquid	56.0		0	87.1	31.1	
		Red blood cells			0.7	12.4		
	Plasma	Supernatant liquid	5.3		0	93.0	87.7	2.0

* Combined selenium = total selenium − (selenate + selenite).

After incubation of the whole blood and blood cells with selenite, considerable hemolysis of the cells occurred, while selenate did not affect the cells. Results of the experiments are given in Table III. The whole blood reduced the selenate to selenite, but the amount of selenite formed was considerably less than with liver. However, "combined selenium" was higher than that observed in the liver experiments.

In order to study the part of the blood responsible for the reduction of selenate, we tested the red blood cells and plasma separately with this compound. The results indicate that the plasma has the ability to reduce the selenate to selenite and volatile selenium, and to change the form of selenium, since 55.6 per cent of selenium recovered was "combined selenium." The red blood cells did not convert the selenate to selenite and only 10.5 per cent of "combined selenium" was recovered.

Similar experiments with the selenite were carried out, the results of which are also given in Table III. The selenite was reduced to elemental selenium by whole blood and by the red blood cells, while the plasma caused no reduction. The distribution of selenite in the supernatant liquid with whole blood, plasma, and red blood cells showed considerable variation. Small amounts of selenite remained in the supernatant liquid with whole blood and plasma, while with the red blood cells 56 per cent of the selenite was unchanged. The supernatant liquid of the whole blood, red blood cells, and plasma contained "combined selenium." The amounts present were as follows: whole blood, 75.0 per cent; red blood cells, 31.1 per cent; plasma, 87.7 per cent.

TABLE IV

Chemical Changes in Sodium Selenate and Selenite in Contact with Beef Spleen at Room Temperature for 2 Hours

Form of Se added	Se recovered from	Form of Se recovered from various fractions				Combined Se*	Volatile Se recovered
		Selenite	Selenate	Elemental	Total		
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Sodium selenate	Supernatant liquid	18.3	42.4	0	62.7	2.0	
	Centrifuged sediment from supernatant liquid			0	2.6		
	Macerated spleen			0.8	22.1		4.2
Sodium selenite	Supernatant liquid	5.0			24.2	19.2	
	Centrifuged sediment from supernatant liquid			1.3	6.1		
	Macerated spleen			19.5	47.3		6.7

* Combined selenium = total Se − (selenate + selenite).

Effect of Spleen on Sodium Selenate and Selenite—The sequence of changes when selenate and selenite were in contact with beef spleen was similar to that observed with the liver, as can be seen from Table IV. This was the only tissue in which we have obtained elemental selenium when we started with selenate. Although the amount was small, it is an indication that after selenite is formed from selenate reduction to elemental selenium can take place.

Treating the spleen with selenite selenium, we obtained 19.5 per cent of elemental selenium. Reduction of selenite to the elemental form with this tissue was greater than with any of the other tissues tested.

Only 5 per cent of selenite, the form added, was present; the remainder was either in combination with tissue proteins or was converted into volatile selenium.

Effect of Guinea Pig Intestine on Sodium Selenate and Selenite—According to our observation, selenite and selenate showed remarkable differences in their physiological action on guinea pig intestines. Using the same method as in the previous experiments, we have investigated the effect of this organ on sodium selenate and selenite.

The results of these experiments are given in Table V. It is interesting to note that the guinea pig intestine did not produce any changes in the form of selenium added, except the attachment of selenium to the tissue.

TABLE V

Effect of Fresh Guinea Pig Intestine on Sodium Selenate and Selenite

Form of Se added	Se recovered from	Form of Se recovered* from various fractions		
		Selenite	Selenate	Total
		per cent	*per cent*	*per cent*
Sodium selenate	Supernatant liquid	0	89.5	90.2
	Guinea pig intestine			10.4
" selenite	Supernatant liquid	66.3	0	67.2
	Guinea pig intestine			33.6

* Elemental, none.

With this tissue as in the previous experiments the selenite showed a greater affinity than the selenate for the tissue. These results indicate that the difference in the physiological action of selenate on guinea pig intestine was due to the failure of this organ to change the selenate to selenite.

DISCUSSION

It is apparent from the data presented here that fresh beef liver, spleen, whole blood, and plasma change the forms of selenium *in vitro*, while autoclaved liver and fresh guinea pig intestine do not produce similar changes. The red blood cells produce changes by forming "combined selenium" when selenate or selenite is added. These results suggest that the fresh guinea pig intestine does not contain the necessary factor or factors (enzymes) to decompose the selenium *in vitro*. The factor or factors (enzymes) in the liver which decompose the selenite are heat-labile, since autoclaving the tissue destroys the substances which brought on the decomposition of the compounds. Selenate is converted into selenite by various fresh tissues. These tissues show a great deal of variation in their ability to change the selenate to selenite. The highest activity is shown by the liver, followed by spleen, whole blood, and plasma. The red blood cells are unable to change selenate to selenite. The attachment of selenite to tissue proteins is evidenced from the results. Only a small amount of selenite was recovered from the supernatant fluid as selenite; the larger amounts were in the centrifuged sediment or tissues, or they combined with the soluble proteins and were recovered as "combined selenium."

The distribution of the selenate and selenite after contact with the various tissues shows some variations, but the trend appears to be similar. These experiments indicate that the metabolism of selenate and selenite is similar, but the amount of volatile selenium formed from the selenite is much greater than from selenate. This may explain the differences in the results obtained by Schultz and Lewis (3) and McConnell (4) in their studies on the respiratory elimination of volatile selenium. The former used selenite and obtained 17 to 52 per cent volatile selenium and the latter used selenate and obtained 3 to 10 per cent of volatile selenium. The amount of volatile selenium formed by the addition of selenite and selenate to the liver tissue approximates the amounts reported *in vivo*. However, the total volatile selenium in all the organs studied was higher than reported by these workers. We must take into consideration that in *in vivo* experiments the volatile substances are transported by the blood and that considerable changes may occur in the volatile substances.

Wright (10) in studying the effect of sodium selenate and selenite on oxygen consumption of mammalian tissues observed that a higher concentration of selenate than selenite was necessary for equivalent depression. He observed that brain slices were little affected by selenate, while the oxygen uptake of liver slices was altered by a lower concentration. Kidney and muscle were slightly more sensitive than brain but less so than liver. Interpreting his results on the basis of our present observations would indicate that the addition of selenate to liver tissue, which in our experiment was able to convert the selenate into selenite, would interfere with the oxygen consumption. After conversion occurred, the effect would be the same as that of equivalent amounts of selenite. Selenite depressed the rate of oxygen consumption in all of the tissues studied. This fact suggests that the toxicity of selenate to the tissue may depend upon the ability of the tissues to convert the selenate into selenite.

The sequence of transformation by the various tissues indicates that the decomposition of selenium follows a definite pattern, from selenate to selenite, "combined selenium," and volatile and elemental selenium. There is a possibility that *in vivo* the reactions are reversible. Observations in animal poisoning seem to indicate this fact. Beath (11) observed that symptoms of poisoning, due to the delayed action of selenium, occurred several months after the animals were removed from seleniferous areas.

SUMMARY

1. In *in vitro* experiments we demonstrated that sodium selenate is converted into selenite and volatile selenium by beef liver, spleen, whole blood, and plasma. These tissues also convert the selenite to volatile selenium and reduce selenium to the elemental form. There are variations in the ability of the tissues to bring about these changes.

2. Autoclaved beef liver and fresh guinea pig intestine are unable to effect any changes in selenate or selenite. The factor or factors present in liver which decompose selenium are heat-labile since they are destroyed by autoclaving. The fresh guinea pig intestine does not contain the factors necessary for the decomposition of the compounds studied.

3. Decomposition of selenate and selenite follows a definite sequence of reactions. Some of the changes in the forms of selenium were determined by chemical means and others by the differences in the results obtained, since no methods at the present time are available for their determination.

4. Recovery of volatile selenium was not quantitative with the absorbents used.

5. On the basis of these experiments we have proved the postulation that selenate and selenite are changed by the tissues and the toxicity of selenate is due to its conversion to selenite by the tissues.

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LETTERS TO THE EDITORS

THE RÔLE OF BICARBONATE IN THE GLUTAMIC ACID METABOLISM OF LACTOBACILLUS ARABINOSUS*

Sirs:

In a study of glutamic acid metabolism, we have found that oxalacetate inhibits the growth of *Lactobacillus arabinosus* when the keto acid is added to the medium devised by Hac, Snell, and Williams.[1] The inhibition is dependent on the pH of the medium. At a concentration of 0.82×10^{-3} M glutamic acid, 24×10^{-3} M oxalacetate is required to inhibit completely at pH 5.7, whereas, at pH 7.7, only 6×10^{-3} M oxalacetate is needed during an incubation period of 20 hours. The inhibition is overcome by a 4-fold increase of glutamic acid (3.2×10^{-3} M) or by a concentration of 7×10^{-5} M glutamine. The effect of glutamine is not a matter of accelerated growth rate, since this is the same in the presence of glutamic acid, glutamine, or glutamine plus oxalacetate. It appears unlikely that oxalacetate itself is responsible for the growth inhibition, for the keto acid decomposes within a few hours[2] even in the absence of bacteria. Malonic acid, fumaric acid, α-ketoglutaric acid, α-hydroxyglutaric acid, citric acid, succinic acid, or pyruvic acid does not inhibit bacterial growth at concentrations equivalent to those of oxalacetate.

The inhibition appears to arise from the carbonic acid formed in the spontaneous breakdown of the oxalacetate; sodium bicarbonate in the same molar concentrations as oxalacetate inhibits bacterial growth. The inhibition is also dependent on pH: complete inhibition occurs at pH 7.4 with 0.8 mg. of sodium bicarbonate per ml. (9.5×10^{-3} M) and at pH 6.3 with 2.8 to 3.6 mg. in the presence of 0.82×10^{-3} M glutamic acid.

The bicarbonate appears to interfere with the transformation of glutamic acid to its amide, for the inhibition is overcome by a 3-fold increase of glutamic acid or by the addition of traces of glutamine (0.4 γ per ml., 2.7×10^{-6} M). The blocking of amidation by bicarbonate is probably of greater biological significance than the interference with glutamic acid metabolism at this stage by the sulfoxide derived from methionine.[3] Since glutamine

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does not undergo transamination,[4] the concentration of bicarbonate may be the determining factor in directing the utilization of glutamic acid either toward amidation or toward transamination. In turn, the concentration of carbon dioxide is regulated by the mechanisms of carbon dioxide fixation, one of the end-products of which is aspartic acid formed by transamination from glutamic acid. The lowering of the carbon dioxide concentration by this process may channel glutamic acid metabolism in the direction of amide formation, thus restricting the utilization of this amino acid for transamination.

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RATE OF PENETRATION OF ELECTROLYTES INTO NERVE-FIBERS

Sirs:

Among the many substances of interest in connection with permeability, which is one of the most essential factors in the function of living cells, ions play a particularly important rôle in the physicochemical mechanism of nerve activity.

The permeability of the surface membranes to ions and their rate of penetration have been investigated with the aid of radioactive ions. Giant nerve-fibers of squid, as used previously for the study of the penetration of anticholinesterases,[1] were exposed for varying periods of time to artificial sea water in which one of the normally occurring ions was replaced by the radioactive ions in equimolar concentration. After exposure, the axoplasm was extruded and the concentration of the radioactive ions determined with the aid of a Geiger counter. So far, K, Na, and Ca have been studied in this way.

The concentration of K in the axoplasm is 18 times as high as in sea water. On exposure to sea water containing radioactive K^{42} in the usual concentration (0.013 M), a high rate of penetration against the gradient was found. Within 60 minutes the radioactive K inside was about twice as high as outside. With a K concentration outside about twice normal, the rate of penetration was approximately doubled. The rate remained relatively high throughout the observation period (100 minutes), although it was highest in the beginning and decreased slowly and continuously.

The concentration of Na is very low in the axoplasm, whereas it is high in sea water (0.52 M). On exposure of the nerves to sea water with Na^{23} partially replaced by radioactive Na^{24}, it was observed that the rate at which Na penetrated was high during the first 15 to 20 minutes but then became low and remained fairly constant. The amount inside rose to about 25 to 30 per cent of that outside.

The concentration of Ca is also high in sea water compared with that in the axoplasm. On exposure of the nerves to sea water containing radioactive Ca^{45} (0.012 M), the Ca penetrated up to a concentration corresponding to about 60 to 80 per cent of that outside. The concentration of the three ions inside (C_i) compared to that outside (C_o) as a function of time of exposure is shown in the figure.

The implication of these results will be discussed in detail elsewhere.

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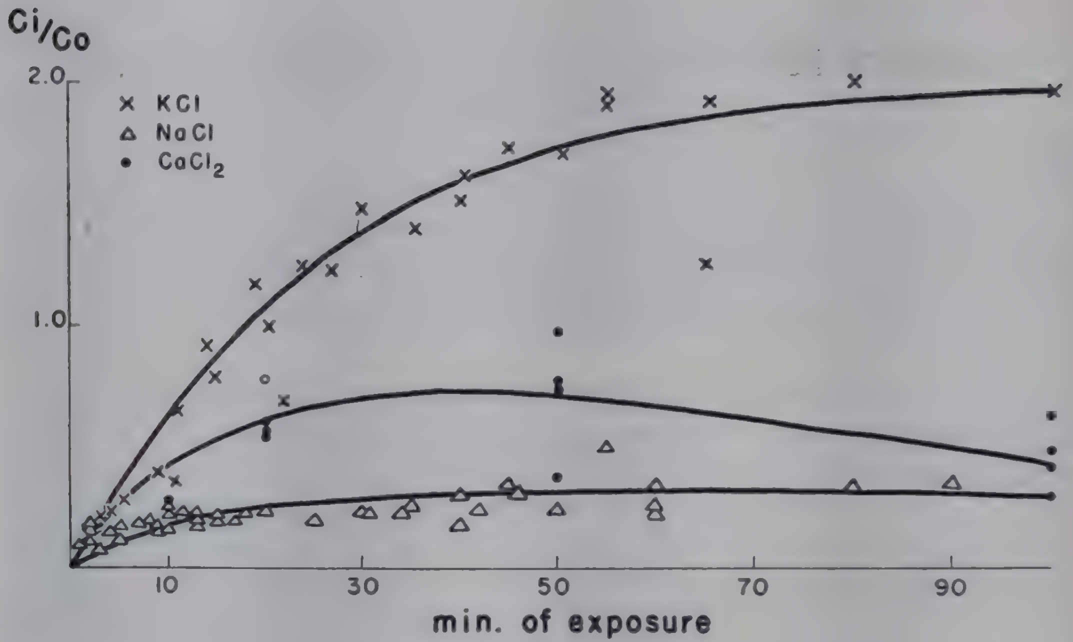
[1] Bullock, T. H., Nachmansohn, D., and Rothenberg, M. A., *J. Neurophysiol.*, **9,** 9 (1946).



They are a striking confirmation of the general conclusion of Krogh[2] and other investigators that permeability is not a simple diffusion but requires an active process in the surface membranes.

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[2] Krogh, A., *Proc. Roy. Soc., London, Series B*, **133**, 140 (1946).

MAGNESIUM AS AN ACTIVATOR OF ANTINVASIN*

Sirs:

Haas[1] showed that there occurred in serum a factor, which he called antinvasin I, that diminished the activity of hyaluronidase on hyaluronic acid. This activity of antinvasin I was followed by viscosimetric techniques.[2] We attempted to use citrated blood plasma in place of serum to inhibit hyaluronidase and were unsuccessful. This led us to the observation that serum was inactivated in its antinvasin potency by the addition of citrate (Experiment 1) or oxalate. Recalcification failed to restore the antinvasin activity, whereas the addition of Mg was completely effective.

Effect of Salts on Antinvasin I Activity

Experiment No.	Serum	Added salt	Final concentration of added salt × 10^{-3}	Antinvasin activity	Per cent of activity
1	Untreated	None		3.09	
	“	Na citrate	0.2	1.85	60
	“	“ “	2.0	1.15	37
	“	“ “	4.0	0.59	19
	“	“ “	8.0	0.21	7
	“	“ “	20.0	0.03	1
	“	“ “	40.0	0	0
	None	“ “	40.0	0	0
2	Dialyzed*	None		0.38	12
	“	MgCl$_2$	0.01	0.64	21
	“	“	0.1	1.20	39
	“	“	1.0	2.22	72
	“	“	10.0	4.24	137
	None	“	10.0	0	0
3	Dialyzed	None		0.38	12
	“	CaCl$_2$	0.3	0.63	20
	“	“	0.6	1.57	51
	“	“	1.0	1.31	42
	“	“	2.0	1.23	40
	“	“	3.0	1.10	36
	None	“	3.0	0	0

* Pig serum was dialyzed against 0.155 M borate buffer, pH 6.7, for 40 hours, followed by dialysis against distilled water for 24 hours.

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* This work was supported in part by grants from the Brush Foundation of Cleveland and by the Research Fund of the Stanford University School of Medicine.

[1] Haas, E., *J. Biol. Chem.*, **163**, 63 (1946).

[2] A purified hyaluronidase was obtained from the Schering Corporation; the hyaluronic acid was prepared from human umbilical cord; pig serum was the source of antinvasin I.

We therefore tested the hypothesis that Mg was an activator of antinvasin by diminishing serum Mg to 0.05 mg. per cent and Ca to 3.4 mg. per cent by dialysis. Dialysis resulted in a loss of activity which could be restored by the addition of Mg (Experiment 2); Ca was relatively ineffective (Experiment 3).

We are now engaged in relating these findings to citrate metabolism. The fact that citrate occurs in prostatic fluid,[3] that hyaluronidase is found in sperm,[4] and that a gel presumably of hyaluronic acid surrounds the ovum[5] suggests the possibility that in the process of fertilization the effectiveness of sperm is dependent upon the citrate content of the prostatic fluid and its protective action against antinvasin in the female reproductive tract.

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Received for publication, November 3, 1947

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[3] Huggins, C., and Neal, W., *J. Exp. Med.*, **76**, 527 (1942).

[4] McLean, D., *J. Path. and Bact.*, **34**, 459 (1931).

[5] McLean, D., and Rowlands, L. W., *Nature*, **150**, 627 (1942).

MILK XANTHOPTERIN OXIDASE AND PTEROYLGLUTAMIC ACID*

Sirs:

Wieland and Liebig[1] have recently described the presence in liver and milk of an enzyme which catalyzes the oxidation of xanthopterin. We have found that xanthopterin oxidase can be concentrated in the following way: Whey is 0.75 saturated with ammonium sulfate. The precipitate is redissolved in pyrophosphate buffer, pH 8.5, and reprecipitated by 0.25 to 0.38 saturation. The product is redissolved in 0.1 M pyrophosphate (pH 8.5) and repeatedly fractionated with ammonium sulfate, the golden brown color, which is apparently associated with the xanthopterin oxidase activity, being employed as a guide. The precipitates so secured are partially bleached in 2 to 4 minutes on addition of xanthopterin under anaerobic conditions; this effect can be measured spectrophotometrically at 470 mμ, at which wave-length neither xanthopterin nor leucopterin absorbs significantly. The decrease in density at 470 mμ amounts to about 25 per cent. The color is restored by oxygen, which, however, causes gradual precipitation of leucopterin.

The catalytic activity of xanthopterin oxidase has been studied by ultraviolet spectrophotometry and by a fluorometric method. These methods have been used to detect and determine xanthopterin in biological materials.

The catalytic activity of xanthopterin oxidase has been studied by two methods: (1) an ultraviolet spectrophotometric method and (2) a fluorometric method. The same two methods have been used to detect and to determine xanthopterin in biological material.

Spectrophotometric Method—When xanthopterin is oxidized in the presence of the specific oxidase, the following changes in density take place: the density at 330 mμ increases markedly and the density at 270 mμ decreases; at 288 mμ no change in density takes place. For each microgram of synthetic xanthopterin[2] per ml. the density at 330 mμ increases by 0.031. The product formed is presumably leucopterin, inasmuch as the ultraviolet spectrum exhibits the characteristics of that substance (density maxima at 340 and 286 mμ). The particularly low solubility of the substance formed as well as the shift in fluorescence with pH points in the same direction.

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* Supported by grants from the Donner Foundation, Inc., the Lederle Laboratories, the Rockefeller Foundation, the Carlsberg Foundation, and the Ella Sachs Plotz Foundation.

[1] Wieland, H., and Liebig, R., *Ann. Chem.*, 555, 146 (1944).

[2] Kindly furnished by Dr. T. H. Jukes and Dr. E. L. R. Stokstad, Lederle Laboratories.

Fluorometric Method—Xanthopterin exhibits a strong blue fluorescence at pH 8 (M/15 phosphate buffer). Upon addition of a small amount of the specific oxidase the fluorescence decreases rapidly. The product formed is practically devoid of fluorescence but can be converted to a fluorescent substance by addition of concentrated NaOH. This reaction is supposed to be characteristic for leucopterin.[3] The enzymic modification of the fluorometric method permits a quite specific estimation of xanthopterin in concentrations as small as 0.01 γ per ml. Urine (directly or as a norit eluate[4]) can be analyzed for xanthopterin by the same method.

Effect of Pteroylglutamic Acid on Xanthopterin and Xanthine Oxidase—Milk xanthopterin oxidase is strongly inhibited by the addition of minute amounts of synthetic pteroylglutamic acid[2] (PGA). Thus, in a sample containing oxidase and xanthopterin (0.06 micromole per ml.) the rate of oxidation was reduced to less than 5 per cent of the original activity by the presence of only 0.05 micromole of PGA per ml. of mixture. Liver xanthopterin oxidase[1] is likewise inhibited strongly by small amounts of PGA. Xanthine oxidase responds the same way, whereas uricase is completely unaffected by addition of PGA.

Pteroic acid[2] exerts a strong inhibition on xanthopterin oxidase. The pterin compound liberated after zinc reduction of PGA[5] possesses only a slight inhibitory effect. Pteroyldiglutamic acid (α-peptide)[2] and pteroyltriglutamic acid (γ-peptide)[2] exert no inhibition.

The marked PGA inhibition of xanthopterin oxidase is an observation which may have bearing upon further studies of the xanthopterin and purine metabolism in the body.

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[3] Decker, P., *Z. physiol. Chem.*, **274**, 223 (1942).

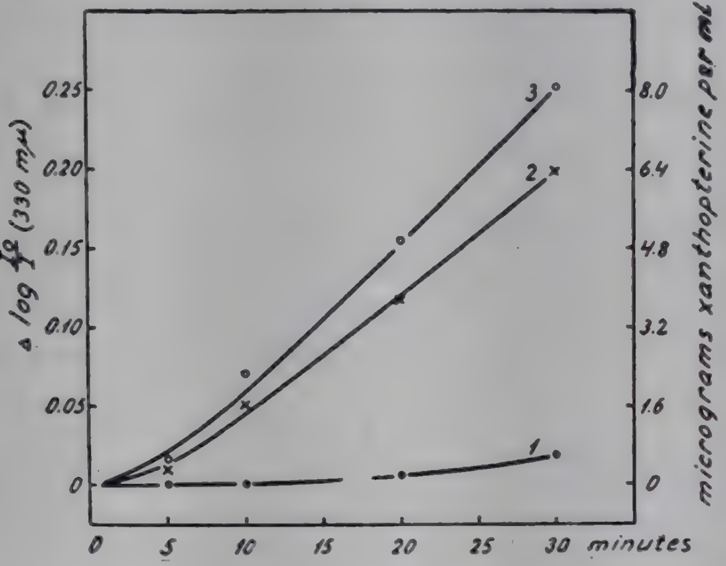
[4] Koschara, W., *Z. physiol. Chem.*, **240**, 127 (1936).

[5] Hutchings, B. L., Stokstad, E. L. R., Boothe, J. H., Mowat, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, **168**, 705 (1947).

ENZYMIC TRANSFORMATION OF PTEROYLGLUTAMIC ACID*

Sirs:

A further study of the previously described inhibition of milk xanthopterin oxidase by pteroyl glutamate (PGA)[1] revealed the presence of another enzyme which brings about a transformation of PGA into a substance which has lost the inhibitory action towards the oxidase.



The figure represents an experiment in which 0.11 micromole of PGA was incubated in 0.1 M pyrophosphate buffer with or without the presence of a concentrated milk xanthopterin oxidase preparation. A sample of the enzyme alone was also incubated. The incubation time was 4 hours at 37°. After the incubation 0.15 micromole of xanthopterin was added to all three samples and xanthopterin oxidase was added to the sample previously incubated with PGA alone (Curve 1). It can be seen that in the sample in which PGA was incubated with the enzyme prior to the addition of xanthopterin (Curve 2) the rate of oxidation proceeds almost as rapidly as in the sample without PGA (Curve 3), whereas PGA incubated in pyrophosphate alone shows the typical marked inhibition of the oxidation (Curve 1).

The enzyme which catalyzes the transformation of PGA into a non-

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* Supported by grants from the Donner Foundation, Inc., the Lederle Laboratories, the Rockefeller Foundation, the Carlsberg Foundation, and the Ella Sachs Plotz Foundation.

[1] Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 349 (1948).

inhibitory substance is fully active under anaerobic conditions. This enzyme has not been separated from the oxidase. It was found, however, to have a pH optimum about pH 8.5, whereas the oxidase has its maximum effect about pH 7.5. The transformed PGA product shows the same ultraviolet spectrum as that of the original PGA.

The transformation is not accompanied by any liberation of *p*-aminobenzoyl glutamate or of free glutamic acid nor is there any increase in fluorescence.

The chemical properties of the transformed PGA are under investigation. Moreover, Dr. Hoff-Jørgensen of the Biochemistry Division has undertaken an investigation of the properties of the transformed PGA as a growth factor towards *Lactobacillus casei* and *Streptococcus faecalis* R. The enzymic transformation of PGA is not accompanied by any loss in growth potency. Further results of this study which is being continued will be published later.

At the present time we are unable to state whether there is any relationship between the enzymic transformation of PGA described here and the action of certain liver fractions (possessing xanthopterin oxidase activity) which are reported to augment the potency of PGA as a hematopoietic agent.[2]

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[2] Jacobson, W., and Good, P. M., *XVII Internat. Physiol. Cong., Abstracts*, Oxford, 157 (1947).

THE SOURCE OF UREA CARBON

Sirs:

Rittenberg and Waelsch[1] and Evans and Slotin[2] have reported experiments designed to test the hypothesis that carbon dioxide is the source of urea carbon. These authors incubated rat liver slices in a medium containing bicarbonate labeled with isotopic carbon and were able to show that under these circumstances at least one-half of the carbon of the urea synthesized originated from the labeled bicarbonate.

We have recently obtained data on the relation of carbon dioxide to urea carbon in the intact animal which are pertinent to the question under discussion. A rat fed L-methionine containing C^{14} in the methyl group[3] continuously oxidized the radiomethyl radical to radiocarbon dioxide[4] throughout a 2 day period, and hence continuously labeled the total carbon dioxide produced by the respiration of its tissues. The specific activity of the expired carbon dioxide was determined for each day of the experiment and compared with the specific activity of the urea excreted on the same day.

The expired carbon dioxide was collected in sodium hydroxide solution, precipitated as barium carbonate, and its radioactivity determined by means of a thin mica window Geiger-Müller counter. The urine was collected each day in 0.1 N sulfuric acid. Aliquots of the daily urine samples were neutralized and digested with urease. The carbon dioxide formed by the hydrolysis of urea was liberated with excess acid and collected in sodium hydroxide solution. The radioactivity was determined as indicated above.

Radioactivity of Carbon Dioxide and Urea Produced by Rat Fed Radiomethyl Methionine

Compound	Specific activity, counts per min. per mg. C	
	1st day	2nd day
Carbon dioxide	468	106
Urea	465	102

The results are shown in the table. Equal specific activities were obtained for the carbon dioxide exhaled and the urea excreted on the same day. The significance of this correlation is enhanced by the equal reduction in the *specific activity* of both the urea and carbon dioxide on the 2nd day of the experiment. Furthermore, from the 1st to the 2nd day there was a 35 per cent increase in urea production and a 13 per cent decrease in carbon dioxide production. These findings indicate that the carbon of urea is quantitatively derived from carbon dioxide.

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[1] Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136**, 799 (1940).

[2] Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 805 (1940).

[3] Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).

[4] Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **169**, 757 (1947).

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CORRECTIONS

On page 310, Table I, column 2, Vol. 171, No. 1, November, 1947, read "*Oxygen consumption, cc. per 100 sq. cm. body surface*" for "*Oxygen consumption, cc. per sq.m. body surface.*"

On page 314, Table III, column 4, opposite "Jejunoileal content," read "*27.20*" for "*6.63.*"

On page 318, line 12, read "*radioactivity in the urine was not extracted*" for "*radioactivity in the urine was extracted.*"

On page 48, Equation 3, Vol. 172, No. 1, January, 1948, read $\frac{dX}{dt} = -\frac{eC}{100}$.

EFFECT OF VITAMIN A SUPPLEMENTS UPON THE STATE OF VITAMIN A IN BLOOD SERUM OF THE DAIRY COW AND IN BLOOD SERUM AND LIVER OF ITS NEONATAL CALF*

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(Received for publication, September 15, 1947)

It has been observed that when cows received concentrates of natural vitamin A ester during the terminal month of gestation their new-born calves possessed greater blood and liver reserves of vitamin A than did calves from dams receiving a standard ration, either with or without the addition of pasture grasses (1). Conceivably the state of this vitamin in the blood serum of the supplemented cows might be different from that normally present. If the placental membranes exhibited differential permeability to the various states of vitamin A in the blood, the form of vitamin A in the ration of the dam might be expected to affect the vitamin reserves of the fetus. The present investigation was undertaken to study the relationship between the form of vitamin A supplement consumed by the pregnant cow and the state of vitamin A in its blood and in the blood and the liver of the new-born calf.

Procedures

Feeding and Management—Sixteen pregnant cows and heifers representing four breeds, Holstein, Ayrshire, Jersey, and Guernsey, were used in this investigation (Table I). Starting 4 weeks previous to expected parturition, they were given daily oral doses of 500,000 I.U. of either the alcoholic or the esterified form of vitamin A, the quantity of supplement being doubled 2 weeks later. These cows were part of a group also used in another study, in which additional details of feeding and management practices have been presented (2).

Collection of Samples—Samples of venous blood were collected at the periods indicated in Table I. Most of the blood samples were collected in the morning, allowed to clot, and centrifuged to obtain serum, which was analyzed immediately. The remaining samples were collected in the afternoon before administering the vitamin supplement. Serum from these samples was stored in the dark at 4° until the following morning, when

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TABLE I

Vitamin A in Blood Serum of Cows Given Supplements of Either Vitamin A Alcohol or Ester during Terminal Stages of Gestation

H, A, J, and G refer to Holstein, Ayrshire, Jersey, and Guernsey breeds, respectively, from which samples were taken.

Oral supplement of vitamin A	Cow No.	Total vitamin A, γ per 100 ml.					Per cent of total vitamin A as ester				
		Period I*	Period II	Period III	Period IV	Period V	Period I*	Period II	Period III	Period IV	Period V
Alcohol	H-137		40	43	26			8	25	15	
"	H-136	30	43	47	30	25	15	24	24	27	10
"	H-115	35	35	37	19		7	18	30	5	
"	A-252	28	46	43	33	43†	7	17	22	14	14†
"	A-243	37	40	44	20	22	7	16	20	5	22
"	J-379	25	45				5	20			
"	J-365	42	40	45	21	24	20	17	40	21	9
"	G-465	23	40	54	21	35	16	8	25	9	1
"	G-452	41	48	43	18	28	24	18	11	11	6
Average		33	42	45	24	27	13	16	25	13	10
Ester	H-132		38	51	16‡	32		10	25	15‡	3
"	H-128	34	38	38	17		2	13	20	0	
"	A-241	25	33	29	23		7	10	14	18	
"	J-378		51	62	31	38		21	33	14	9
"	J-358	41	43	34	22	18	9	21	18	5	9
"	G-468	47	52	67	32		10	13	32	14	
"	G-453	35	37	44	24		6	23	20	22	
Average		36	42	46	25	29	7	16	23	12	7
" of both groups		34	42	45	24	28	10	16	24	13	9

* Period I, previous to supplementation with 500,000 I.U. of vitamin A daily, approximately 4 weeks ante partum; Period II, cows given 500,000 I.U. of vitamin A daily for 8 to 18 days, approximately 2 to 3 weeks ante partum; Period III, cows given 1,000,000 I.U. of vitamin A daily for 1 to 2 weeks, 2 to 15 days ante partum; Period IV, 0 to 2 days post partum, supplement discontinued at parturition; Period V, during the period of normal milk production, at least 2 months post partum, some of the cows were grazing on pasture grasses at this time.

† 3 weeks post partum; not included in the averages.

‡ 4 days post partum; not included in the averages.

analyses were made. Blood samples from calves were treated the same as those from cows except that, in some cases, it was necessary to postpone analyses of the samples until the 2nd day after collection.

A number of the male calves were sacrificed to obtain livers, which were analyzed for total vitamin A and for the alcohol-ester distribution; the analyses were carried out either immediately after removal of the livers or after retaining them in a frozen state for not more than 6 days. The state of vitamin A also was determined in both the liver and the blood of several male calves from dams that were subjected to the regular herd régime.

TABLE II

Vitamin A in Blood Serum and in Liver of Dairy Calves

H, A, J, and G refer to Holstein, Ayrshire, Jersey, and Guernsey breeds, respectively, from which samples were taken.

Supplement of dam	No. of dam of calf	Blood serum			Liver		
		Time of sample after birth	Total vitamin A	Total vitamin A as ester	Time of sample after birth	Total vitamin A	Total vitamin A as ester
			γ per 100 ml.	*per cent*		*γ per 100 ml.*	*per cent*
Vitamin A alcohol	A-243	Immediately	18	92	Immediately	15	72
" " "	G-452	"	5	16	1 hr.	17	80
" " "	A-252				1 "	10	70
" " ester	H-378	2 hrs.	6	16	2 hrs.	13	83
" " "	A-241				Immediately	5	71
" " alcohol and tocopherols	G-459	1 hr.	7	12	2 hrs.	12	71
" "	J-374	1 "	9	10	2 "	12	86
" "	G-458				1 hr.	17	84
5 gm. tocopherols	A-240	Immediately	5	25			
None	A-230				1 hr.	1	84
10 gm. mixed tocopherols	H-138*	10 hrs.	8	61	10 hrs.	1	80
" "	A-200†	4 days	17	26	4 days	7	74
Vitamin A ester	H-128†	4 "	22	22	4 "	30	73
None	H-126‡	4 "	11	26	4 "	2	77
Vitamin A ester	J-358§				39 "	13	82

* Calf given one feeding of 5 pounds of colostrum 4 hours after birth.

† Calf fed colostrum from its own dam.

‡ Calf fed milk from its own dam, which was milked through gestation without a dry rest period.

§ Calf weak and died at 39 days of age.

The experimental treatment of each of the calves and the postnatal age of the calf at which materials were taken for analyses are shown in Table II.

Method of Analysis for Blood—The vitamin A alcohol and ester content of blood serum was determined by a procedure similar to that developed for milk and colostrum (3). The following modifications were found desirable in applying the method to blood serum. Instead of saponifying the whole sample employed for determining total vitamin A, the lipides were extracted from the serum before saponification. For the first extraction, 10 ml. of serum were placed in a glass-stoppered centrifuge tube, mixed with 10 ml. of ethanol, and shaken for 2 minutes with 20 ml. of diethyl ether. Only 10 ml. of ether were used for the second and third extractions, respectively. Previous to the chromatographic phase of the analysis, 5 ml. of purified Skellysolve B (4) were added to the combined ether extracts to reduce the water content. These extracts were allowed to remain undisturbed for 15 minutes, and the water which separated was drained completely. This was followed by removal of the solvent by suction and heating at 60°, dissolution of the residue in purified Skellysolve B, and chromatographic separation of the alcoholic and esterified forms of vitamin A. Vitamin A ester in extracts of blood serum seemed to pass through the chromatographic column somewhat more slowly than did extracts from either milk or colostrum; accordingly, about 20 ml. more of 4 per cent acetone-Skellysolve B mixture were used for elution of the vitamin A than were used for milk and colostrum samples.

The part of the sample to be used for determining the total vitamin A was saponified with a mixture of 8 ml. of alcoholic KOH (5) and 1 ml. of water; only 5 ml. of alcoholic KOH were used for saponification of chromatographed samples. If the alcohol content of the saponification mixture exceeded 90 per cent, artifacts were formed, causing the results of the vitamin A determinations to be too high. Under the conditions employed, it was found possible to overcome most of the difficulty; the average artifact was found to be equivalent to approximately 1 γ per 100 ml. of serum (6). However, inattention to this detail may cause considerable error, especially in the measurement of esterified vitamin A, since it usually is of low concentration. Serum from calves was handled similarly to that from cows, except that the frequent occurrence of samples low in vitamin A made it desirable to double or triple the quantities of serum used.

Method of Analysis for Liver—Whole fresh livers or frozen livers, which were allowed to thaw for 1 day at 4°, were homogenized in a Waring blendor. 20 gm. of each homogenized sample were dried by grinding with anhydrous sodium sulfate. The lipides were extracted from the dried material by agitation in a Waring blendor for 4 minutes with 25 ml. of ethanol and 100 ml. of diethyl ether. This was followed by three 2 minute extractions with 60 ml. of ether each time. A final extraction was made with 30 ml. of ether and 30 ml. of Skellysolve B. After each extraction, the solution was separated from the solids by filtering under suction. The solution containing the extracted material was washed with water to remove alcohol. Aliquots of the extract equivalent to either 2 or 4 gm. of liver were used for analysis. The remainder of the method was similar to that employed for blood and for colostrum. The final extract was adjusted to a volume of 25 or 50 ml., of which 10 ml. were used for determining vitamin A and carotenoids.

Precision of Method—Vitamin A alcohol added to extracts of blood serum previous to chromatography was as completely removed by the adsorbent as was that used in studies on milk and colostrum (3). However, the recovery of added vitamin A ester averaged 87 per cent, which was not so high as was found in the previous work. Since it was observed that when solutions of concentrates of vitamin A ester were chromatographed a second time a small amount of the alcoholic form of vitamin A apparently was retained on the column, it is suggested that the first passage through the adsorbent might have sensitized the ester, causing some of the molecules to undergo a change during subsequent treatment. No correction was made for loss of esterified vitamin A on the adsorbent, since for many samples the loss was approximately of the same magnitude as small apparently unavoidable artifacts, which were discussed in a foregoing section, that usually arose from the saponification procedure. If it is assumed that each sample, those for total vitamin A as well as those for determining the concentration of the esterified form, contained artifacts equivalent to 1 γ of vitamin A per 100 ml. of serum, the corrected vitamin A ester content would average only 3 per cent lower than the values reported here. Hence, the presence of such artifacts made little difference in the over-all results of this study.

The differences in duplicate determinations of samples for total vitamin A averaged ±2.2 per cent of the means of the respective samples. Samples containing only the esterified vitamin A were more variable. This was to be expected, since the vitamin A content of these samples usually was low and the samples were subjected to a greater number of operations in the analytical procedure.

Results

State of Vitamin A in Serum from Cows—Although considerable individual variation was observed, one of the salient findings was the generally low percentage of vitamin A ester in blood serum of dairy cows (Table I). During the periods of vitamin A supplementation and immediately thereafter (Periods II, III, and IV), the percentages of the total vitamin A of the serum occurring as the ester were essentially the same for the experimental group receiving supplements of the alcoholic form of vitamin A as for the group receiving the esterified form. However, as the vitamin

A of the blood serum reached higher levels upon prolonged supplementation with large amounts of either the alcoholic or esterified form of vitamin A,[1] an increase in the percentage of the esterified form was observed. Before supplementation with vitamins was commenced (Period I), an average of 10 per cent of the vitamin A of blood serum occurred as the esterified form, but upon supplementation with 500,000 I.U. of vitamin A for 8 to 18 days (Period II), an average of 16 per cent of ester was found. After increase of the supplement to 1,000,000 I.U. (Period III), 24 per cent of the total vitamin A occurred as the ester. From Periods I to III the alcoholic fraction of vitamin A increased by an average of 4 γ (13 per cent of the initial value), whereas the ester fraction increased 7.3 γ (210 per cent). This indicated that, following supplementation, most of the increase of total vitamin A occurred in the ester fraction.

After parturition (Period IV), when the vitamin A content of blood serum decreased even though supplements of this vitamin had been given ante partum (7), the esterified form averaged 13 per cent of the total vitamin A. 2 months later, when the cows were in normal milk production and some of them had access to pasture grasses (Period V), the ester constituted 9 per cent of the total vitamin A in the blood serum. By application of the t test (8) the differences in the concentrations of vitamin A ester of successive periods were significant ($P = 0.05$ or less) for all except the change from Periods IV to V. The smaller number of determinations and the rather wide variability possibly account for non-significance in the latter case.

State of Vitamin A in Serum and Livers of Calves—Except in the cases of Calves A-243 and H-138, the vitamin A of the blood serum was predominantly in the alcoholic form, only 10 to 26 per cent of the total being esterified (Table II).[1] Similar results were obtained in samples taken before colostrum consumption and in those taken after the calf had received colostrum from its own dam for 4 days.

The blood serum of Calf A-243 contained 92 per cent ester, a value considerably higher than that found in samples taken from other new-born calves prior to colostrum ingestion. Additional observations suggesting an abnormal condition with this animal were a high content of total vitamin A, an unusually high lipide content of the blood, and the highest tocopherol level found for a new-born calf (9). The blood serum of Calf H-138 at birth contained a total of 4 γ of vitamin A per 100 ml. This calf was given 5 pounds of colostrum, a rich source of vitamin A ester (3), and was sacrificed during the period of active digestion and absorption, 6 hours after feeding. At this time, 8 γ of total vitamin A per 100 ml. of serum were found, 61 per cent being in the esterified form.

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[1] Forthcoming reports will consider in greater detail the effect of the vitamin A content of the diet upon the concentration of vitamin A in the blood serum of dairy cows during the terminal stages of gestation and the initial stages of lactation and upon the placental transfer of vitamin A to the blood and liver of the neonatal calf.

Regardless of age of the calf or of the form of vitamin supplement given the dam during the terminal stages of gestation, the vitamin A of calf livers was predominantly in the esterified form; all values were between 70 and 86 per cent (Table II).[1]

Pigments of Blood Serum and of Liver—Since the alumina column separates the carotenoid pigments in a manner similar to the separation of the two forms of vitamin A, this study has provided, incidentally, additional information of interest. The carotene fraction included all pigment that was eluted from the alumina column by 4 per cent acetone in Skellysolve B and which remained in the non-saponifiable matter after subsequent saponification. The carotenoid pigments of the serums (pigments of the non-saponifiable fraction) from cows averaged 97 per cent carotene. This value is in close agreement with the findings of Gillam and El Ridi (10) that most of the pigment of blood serum was carotene, lutein constituting only about 5 per cent of the total carotenoids.

The unsaponified pigments obtained by extracting the serum of certain cows (for the most part those of the Guernsey breed) contained detectable quantities of yellow pigments which were strongly adsorbed at the top of the alumina column. Apparently most of these substances were either destroyed or removed during the saponification and subsequent treatment of the samples from which total pigment concentration was determined (6).

The carotenoids of the blood serum of the four calves sampled after colostrum feeding averaged 92 per cent carotene, a value that is not appreciably different from that obtained for serum from cows. Since so little yellow pigment is present in the non-saponifiable fractions of extracts of liver and of blood of the calves at birth, the degree of precision of the method developed for vitamin A work was not sufficient for satisfactory determinations of the carotenoid-carotene ratios. However, chromatography indicated that the larger part of the pigment of the non-saponifiable matter was carotene. When unsaponified calf liver extracts were chromatographed, a trace of yellow pigment passed through the column at a more rapid rate than was usually observed for carotene. Identification of the pigment has not been undertaken. A strongly adsorbed yellow pigment was observed at the top of the alumina column.[2] If liver extracts are incompletely saponified and washed, some of this pigment may be carried into the final extract and may cause variable results in the determination of the carotene content of liver.

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[2] This pigment was soluble in a water-alcohol mixture, turned orange upon treatment with antimony trichloride reagent, and no longer was extractable by ether or Skellysolve after saponification.

DISCUSSION

The results of this study are in agreement with those of Hoch and Hoch (11) who found that human serum normally contains 10 to 17 per cent vitamin A ester and that doses of either the natural ester as found in liver oil or of preparations of vitamin A alcohol increased the proportion of vitamin A ester in the serum. Clausen *et al.* (12) reported that almost all vitamin A normally was present in human blood in the alcoholic form, and that an increase in the ester fraction occurred when large doses of either free or esterified vitamin A were given by mouth. According to these authors, the increase in the latter form is presumed to result from the absorbed vitamin being transported to storage tissues in the "inactive" esterified state. The increase of the ester fraction herein reported for cows was not so large as for human subjects. This possibly resulted from differences in the levels at which the supplements were given, from differences in the interval between feeding of supplements and collecting of blood samples for analysis, or from differences in the types of digestive system possessed by the respective species. Vitamin A administered to the experimental cows probably passed first into the rumen and subsequently through the other gastric compartments into the small intestine. Hence, as a result of a greater possibility of destruction or of a gradual passage of portions of ingested supplements to the intestine, it is probable that the concentration of vitamin A in the gut of the cow was less than would be the case with monogastric species, such as humans. If vitamin A is transported to the liver as an ester (12), the level of this form of the vitamin in the blood would be expected to be proportional to the rate and degree of absorption.

Another possibility indicated by the results is a direct relationship between the level of total vitamin A of the serum and the proportion present as the ester. In the first place, if the data from all cows are analyzed for the periods vitamin A supplements were given, the product moment correlation (r) between vitamin A content and per cent as ester is 0.58, the correlation being highly significant (8). Secondly, a graphic analysis of the data for blood serum, in a manner similar to that employed by Glover *et al.* for livers ((13) Fig. 1a), indicates that a level of about 10 γ of vitamin A per 100 ml. of serum must be reached before any ester is found. Until additional information is obtained, it will not be possible to explain definitely the experimental observations of increased amounts of the ester following supplementation of the dairy ration with vitamin A.

Although there was no evidence that gestation and parturition affected the vitamin A alcohol-ester ratio, nothing specific was revealed concerning this relation, inasmuch as the present study was not designed to gather information relative to these factors.

When saponification was omitted, Boyer *et al.* (14) observed that their carotene precipitation method yielded vitamin A values 87 to 95 per cent of those found following saponification; hence they reported that vitamin A was present in cattle blood in the alcoholic form. The finding of varying amounts of vitamin A ester in the serum samples of the present study introduces the possibility that the lower values which Boyer *et al.* found were due to a small quantity of esterified vitamin A that was precipitated with carotene by their method. This view-point is supported by the fact that vitamin A has been recovered from filter papers containing the material precipitated by the method of Boyer *et al.* (6).

The blood of calves at birth contained vitamin A in essentially the same state as that of their dams. The results for calves, contrary to indications obtained for the serum of cows, did not suggest that a certain level of vitamin A was reached in the blood before the vitamin appeared as the ester form. There appeared to be a tendency for the concentration of vitamin A alcohol to increase in the calf liver as the total vitamin A of the blood serum increased. This observation was in agreement with the report by Glover *et al.* (13) from rat studies, but since only a limited number of experimental animals was used in the present study, a definite conclusion concerning this relationship is not justified.

The state of vitamin A in the livers of calves (Table II) is similar to that reported for the livers of other animals. However, the percentage of vitamin A ester is slightly lower than noted for rats (13, 15), for dogs (16), and for fish liver oils (17–20), but averaged somewhat higher than the values which Sobotka *et al.* (21) found for fish liver oils using a fluorescence technique. The ingestion of a single feeding of colostrum, rich in vitamin A, apparently did not cause a large temporary increase of the amount of vitamin A alcohol in the liver of Calf H-138, as was observed when guinea pigs were fed large doses of this vitamin (22).

Though the data are limited, observations on the amounts of the esterified form of vitamin A in the blood serum of calves after colostrum consumption suggest that the stage of absorption or the age of the calf is involved. The high proportion of vitamin A ester in the blood serum of the neonatal calf, No. H-138 (Table II), indicates two possibilities: either direct absorption of the colostral vitamin A without hydrolysis, similar to the apparent absorption of unaltered protein by the new-born (23, 24), or rapid absorption and transport of reesterified vitamin A to the storage tissues (12, 25). The percentage of esterified vitamin A in the blood serum of the calves 4 days old (Table II) was considerably lower than that noted for Calf H-138. Factors that might have contributed to these differences are possible alterations in the permeability of the intestines and extension of the time after feeding at which blood samples were collected, which was 6 hours for Calf H-138 and 12 hours for the older calves. The effect of the stage of absorption on the concentration of the ester form of vitamin A is a phase of the problem that warrants additional study.

It was suggested (1) that perhaps the larger vitamin A reserves of calves from dams fed supplements of esterified vitamin A might have resulted from an increase of this form of the vitamin in the maternal circulation, assuming that the placenta either may be more permeable to, or have a lower threshold for, esterified vitamin A. While the change in the ester-alcohol ratio of the maternal blood was not so great as expected, most of the increase was in the ester fraction; hence the present results are in accord with the foregoing conjecture. Supplementation of the diet of the preparturient cow with either form of vitamin A effected a similar increase in the vitamin A ester of the maternal blood and in the total vitamin A reserves of the neonatal calf. Although the present observations seem to be in harmony with the theory (1), further clarification requires additional information on the physiological processes involved.

SUMMARY

Vitamin A in the blood serum of dairy cows was found to be largely in the alcoholic form. Before supplementation of the ante partum ration with either the alcoholic or esterified form of vitamin A, the esterified form averaged 10 per cent of the total vitamin A of the blood serum, but during oral administration of 1,000,000 I.U. of vitamin A daily, the esterified form increased to 24 per cent of the total vitamin A. Most of the increase of total vitamin A of the blood serum occurred in the ester fraction.

The vitamin A of blood serum of calves under normal conditions, both at birth and at 4 days of age, was found to be predominantly of the alcoholic form, while the vitamin A of the livers was mostly of the esterified form.

Carotene was found to constitute about 97 per cent of the carotenoids of the non-saponifiable extract of the blood serum of dairy cows. Carotene constituted about 92 per cent of the total carotenoids of the non-saponifiable extracts of blood serum of calves that was sampled after colostrum consumption. Although most of the pigment of blood serum taken from calves at birth appeared to be carotene, the method used did not provide for a specific determination of the carotene-carotenoid ratio.

Alcoholic and esterified forms of vitamin A given orally to the dam during the terminal stages of gestation seemed to have a similar effect for fortifying the vitamin A reserves of the neonatal calf.

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A TURBIDIMETRIC METHOD FOR THE ASSAY OF HYALURONIDASE

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A variety of methods have been devised for the assay of the enzyme hyaluronidase. These may be divided into four types: (*a*) measurement of the "spreading effect" in the skin of rabbits and guinea pigs (1, 2), (*b*) reduction of viscosity (3, 4), (*c*) prevention of mucin clot formation (5), and (*d*) chemical measurement of total reducing substances, N-acetylglucosamine, or glucuronic acid liberated on the complete hydrolysis of hyaluronic acid (6, 7).

The first group of methods ("spreading activity") is most sensitive, but is time-consuming, requires large numbers of animals, and lacks specificity in that substances other than hyaluronidase have been shown to possess spreading activity. They are of comparatively little value for the routine assay of large numbers of preparations. The viscosity method has been used widely with considerable success. It is, however, tedious and time-consuming and requires large amounts of hyaluronic acid.

The original mucin clot prevention method is based on the fact that hyaluronic acid reacts with acidified albumin to form a clot (5). If hyaluronic acid is first acted on by hyaluronidase, such a clot does not form. This then affords a basis for the estimation of hyaluronidase.

It has been noted that when the smallest amount of enzyme necessary to prevent clot formation is used there remains a uniform turbidity. If larger amounts of enzyme are used, this turbidity does not develop.

The use of the various chemical methods is open to serious question in that Hahn (8) has recently shown that highly purified hyaluronidase does not bring about the complete hydrolysis of hyaluronic acid. The release of glucuronic acid and N-acetylglucosamine apparently depends upon the presence of contaminating enzymes in the crude preparations.

A method based on turbidity development seemed most likely to be rapid, simple, and accurate. Seastone (9) showed that under certain conditions turbidity is directly proportional to hyaluronic acid concentration. Kass and Seastone (10) used this fact in developing a quantitative turbidimetric method for the assay of hyaluronidase. They utilized acidified serum for turbidity development. Leonard, Perlman, and Kurzrok (11) have developed a similar method for the determination of hyaluronidase in semen by use of acidified serum.

It is the purpose of this communication to describe a modified turbidity method with crystalline horse serum albumin. The reagents used in this method are stable and easily reproducible. Exact conditions for the use of this method have been determined.

The turbidity method may be applied to the estimation of hyaluronidase in two ways. The first of these consists of the determination of the rate at which hyaluronic acid is hydrolyzed. This is analogous to the viscosity-reducing method. We have shown that this method is adequate and can be used in kinetic studies (12). It has the disadvantage of requiring a number of different determinations for each sample analyzed and is thus time-consuming. A simpler method consists of determining the amount of hyaluronic acid which remains after some specific time. This requires only one turbidity determination for each preparation being analyzed. The method described in this communication depends on such an estimation.

Materials

Hyaluronic Acid—Human umbilical cords were washed free of blood and stored under acetone in the cold. The acetone powder was prepared by grinding in a meat grinder and washing with acetone. 200 gm. of dry powder, 1200 ml. of Hayem's solution, and 3000 ml. of water were stirred vigorously for 2 hours. The resulting solution was centrifuged and filtered through glass wool. Hyaluronic acid was precipitated by pouring the solution into 12 liters of cold acetone. The stringy material was washed three times with cold acetone, filtered, washed twice with alcohol and anhydrous ether. It was finally desiccated over P_2O_5 for 24 hours. This procedure gave a yield of 6 per cent of a product which gave a relative viscosity of 3.0 at a concentration of 10 mg. per ml. For the turbidity assay it was made up to 3.0 mg. per ml. in a 0.3 M phosphate buffer at pH 5.5.

The solution so prepared is slightly opalescent, but can be obtained water-clear by filtration through a Seitz filter. It is diluted to give a standard turbidity. This usually requires about a 2:3 dilution.

Acidified Horse Serum Albumin—Crystalline horse serum albumin was prepared by the method of Kekwick (13). 1 gm. of purified albumin was dissolved in 1000 ml. of 0.1 M acetate buffer of pH 4.1 and the pH subsequently adjusted to 3.75 with 4 N hydrochloric acid. This solution keeps indefinitely at 4°. Fraction V of bovine albumin, obtained from Armour and Company, was found to work equally well.

Enzyme—Testicular hyaluronidase was prepared from beef testes by the method of Hahn (8) and for the most part the material carried through the first ammonium sulfate precipitation was used. Enzyme so prepared was dialyzed free of sulfate at 4°, lyophilized, and stored in the dry state.. No evidence of loss of activity has been observed in 6 months storage at −20°. For use it was dissolved in 0.2 M borate buffer of pH 7.5. The activity of such preparations was approximately 250 viscosity-reducing units per mg. of N.

Activity of enzyme was ascertained according to Haas (4) and expressed as the reciprocal of the half life × 10^3. All determinations were carried out at 38°. Difficulty was encountered in attempting to standardize this unit, since it was found that the activity of the enzyme varied with different hyaluronic acid preparations. This was circumvented by correcting all activities to that obtained with a given lot of hyaluronic acid. In order to prevent undue introduction of new units in the literature, this same amount of enzyme was used as the arbitrary unit in the turbidity method to be described. The unit to be used in this discussion is then essentially based on a given amount of standard enzyme preparation.

Procedure

1 ml. of enzyme solution is mixed with 1 ml. of hyaluronic acid solution. (The enzyme has usually been made up in 0.5 ml. of 0.2 M borate buffer, pH 7.5, and mixed with 0.5 ml. of 0.9 per cent saline. The saline has been introduced into the procedure for convenience in the determination of hyaluronidase inhibitor.) This mixture is incubated for 45 minutes at 38° in Evelyn cuvettes. At the end of this time 10 ml. of acidified albumin reagent (at room temperature) are added and the mixture is shaken to insure complete mixing. Exactly 5 minutes later (by stop-watch) the tube is read in the Coleman junior spectrophotometer at a wave-length of 600 mμ. Lower wave-lengths can be used, but 600 mμ was selected to obviate interference of hemolysis in the determination of hyaluronidase inhibitor.

All determinations are done in duplicate. The tubes used are carefully selected so as to have a maximum variation of 0.5 of 1 scale division.

EXPERIMENTAL

Relationship of Optical Density to Hyaluronic Acid Concentration—Seastone (9) originally reported that the turbidity produced by a mixture of hyaluronic acid and acidified albumin is directly proportional to hyaluronic acid concentration. That this is so is confirmed by the results illustrated in Fig. 1. It will be seen that the relationship is linear up to about 2.0 mg. and beyond this the curve begins to decrease in slope. At higher concentrations a precipitate and finally a clot form.

Effect of pH on Turbidity Formation—The next group of experiments was performed to determine the optimum pH for the precipitation of the hyaluronic acid-albumin complex. A series of reagents was made up at different pH levels and the turbidity produced was determined.* All other conditions were kept constant in this experiment. The pH value given is that of the final mixture. It will be seen in Table I that turbidity development (expressed as optical density) is maximum at pH 3.82. This is achieved when the albumin reagent is adjusted to pH 3.75. If the pH is above or below this value, the turbidity is markedly decreased.

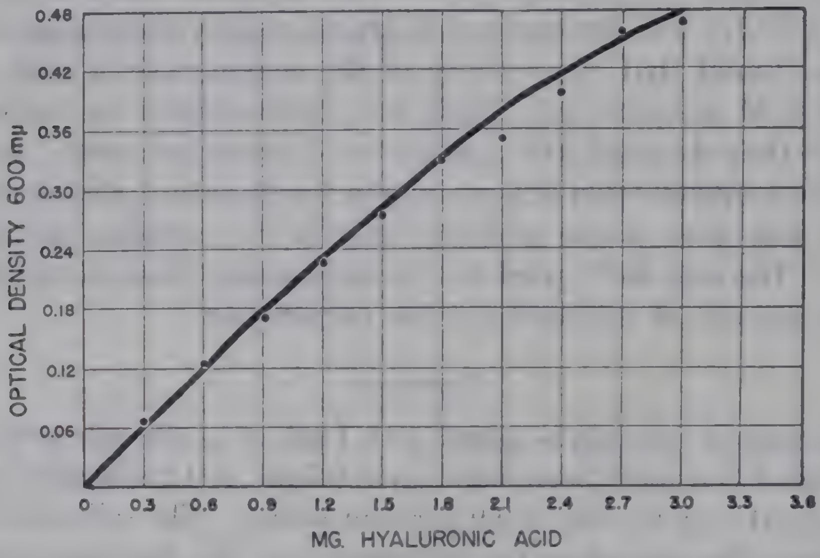

FIG. 1. Relationship of hyaluronic acid concentration to optical density

TABLE I

Effect of pH on Turbidity Development

pH	2.15	3.10	3.42	3.82	4.20
Optical density	0.153	0.274	0.293	0.337	0.305

TABLE II

Effect of Variation of Ionic Strength on Turbidity Development

Ionic strength	0.15	0.25	0.35	0.45
Optical density	0.321	0.248	0.0303	0.0304

Effect of Ionic Strength on Turbidity Development—It was found that turbidity development varied considerably with salt concentration. Table II shows the results of an experiment designed to assess the importance of this factor. It will be noted that the lowest ionic strength used was 0.15. It is inconvenient to go below this value and obtain proper conditions for the maintenance of pH both for enzymatic action and turbidity development. It is evident that the lowest salt concentration produced the maximum turbidity. As salt concentration increases, there is a marked drop in turbidity. The added salt was sodium chloride. No experiments were performed with other salts. As in previous experiments, all other conditions were kept constant.

Effect of Time of Reading on Turbidity—It was noted that turbidity decreases with time. Table III illustrates an experiment in which this factor was quantitatively evaluated. The first reading was taken at 5 minutes since this is the shortest interval that is convenient when a series of tubes is to be read. After longer intervals the rate of change decreases. Routine use of longer intervals was, however, found to be disadvantageous, since the differences in optical density between different concentrations of hyaluronic acid are decreased. It will be noted that the difference in optical density between 1.80 and 0.60 mg. of hyaluronic acid is 0.215 at 5 minutes, while at 30 minutes this had decreased to 0.172. This considerably decreases the sensitivity and range of the method.

TABLE III

Effect of Time on Turbidity Development

Time	Hyaluronic acid concentration			
	1.80 mg.	1.40 mg.	1.00 mg.	0.60 mg.
	Optical density			
min.				
5	0.347	0.270	0.204	0.132
10	0.325	0.262	0.197	0.129
15	0.314	0.260	0.187	0.116
20	0.308	0.258	0.184	0.116
30	0.288	0.235	0.177	0.116

Effect of Albumin Concentration on Turbidity Development—Since samples to be assayed may contain albumin, it was necessary to be sure that the albumin in the reagent is present in excess. This is particularly true when this method is applied to the assay of hyaluronidase inhibitor in blood serum. Fig. 2 shows the turbidity obtained with varying concentrations of albumin. It was found that the turbidity developed approaches a maximum at about 0.50 mg. per ml. It was decided to use 1.0 mg. per ml. routinely in order to assure an excess.

Relationship of Turbidity to Enzyme Concentration—In order to eliminate the necessity of multiple determinations, a method was devised, based on one turbidity determination. The experiment illustrated in Fig. 3 shows the relationship between turbidity (optical density) and enzyme concentration when enzyme has been incubated with hyaluronic acid for 45 minutes.

It is seen that between 0 and 4 units this relationship is essentially linea
The region of linearity can be varied by changing the length of incubation

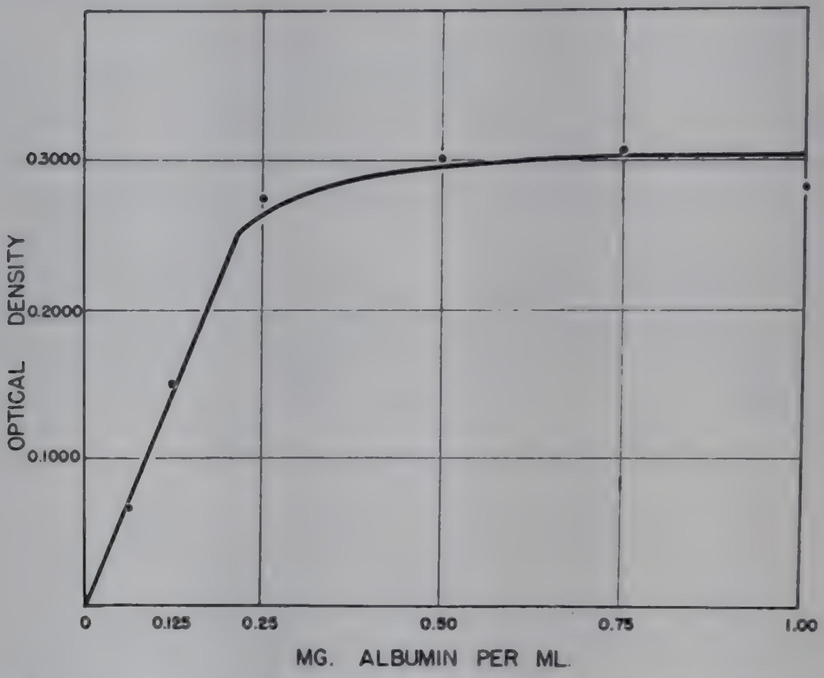

FIG. 2. Relationship of albumin concentration to optical density

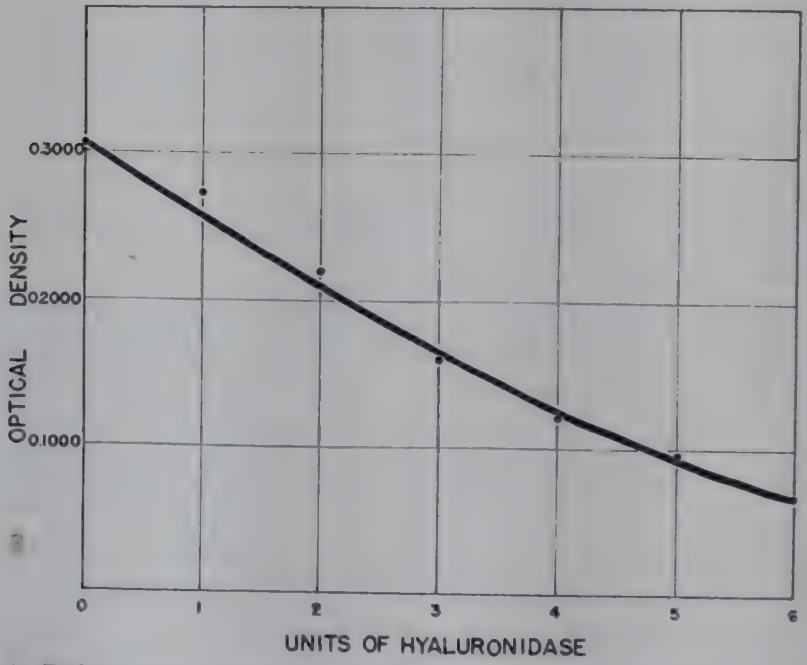

FIG. 3. Relationship of hyaluronidase concentration to optical density

With longer time intervals the method becomes more sensitive with less range, while with shorter time intervals the method becomes less sensitive but with greater range.

Careful attention must be paid to details of pH, salt concentration, and time. Different batches of hyaluronic acid vary in purity, but can be used to get comparable results simply by adjusting the concentration to a given optical density.

Effect of pH on Activity of Hyaluronidase—The next group of experiments was performed to determine the effect of variation of pH on the activity of hyaluronidase under these conditions. Several investigators have shown that the effect of salt concentration varies with pH and conditions of assay (3, 5). A series of phosphate buffers (0.3 M) was made at various pH levels and comparison of activity was made. All other conditions were kept constant. Table IV illustrates the results of these experiments. The k_{v_0} shown was calculated according to a method devised in this laboratory (12). It will be noted that the peak is obtained in the region of pH 5.5.

TABLE IV

Effect of pH on Activity of Hyaluronidase

pH	4.40	5.00	5.58	5.92	6.47
k_{v_0}	0.0281	0.0316	0.0340	0.022	0.016

TABLE V

Effect of Ionic Strength on Hyaluronidase Activity

Ionic strength	0.15	0.21	0.26	0.31	0.36
	Optical density				
Initial turbidity	0.280	0.286	0.299	0.297	0.292
2 units (45 min.)	0.161	0.171	0.194	0.208	0.233
4 " (45 ")	0.071	0.089	0.122	0.140	0.168

It is of interest that activity drops more rapidly on the alkaline side of the maximum than on the acid side. This pH (5.5) was then adopted as the standard in subsequent studies.

Effect of Ionic Strength on Hyaluronidase Activity—Several investigators have studied the effect of variation of salt concentration on hyaluronidase activity. This was found to vary with pH. It seemed desirable to redetermine this by the method described in this paper. For this purpose a series of hyaluronic acid solutions in phosphate buffers of varying ionic strength at constant pH was used. All solutions were adjusted so that the optical density of the turbidity developed was constant. All other solutions were as described under "Procedure." Table V shows the results of such an experiment. In this only one reading was made; namely, after 45 minutes with 2 and 4 units. The results are expressed in terms of optical density and final ionic strength of the reaction mixture. Thus the borate buffer, sodium chloride, and phosphate buffer are included in calculation o the ionic strength. It will be noted that increasing ionic strength causes decrease in the activity of the enzyme in the range studied. It is impractical to use an ionic strength below 0.15 and have suitable conditions for th maintenance of pH. When ionic strength was varied by using a constan amount of phosphate and varying amounts of sodium chloride, simila results were obtained.

It was decided to use a total ionic strength of 0.26 (hyaluronic acid i made up in 0.3 M phosphate buffer). Although this does not represent the

TABLE VI

Reproducibility of Method

Experiment No.	Enzyme units			
	0	2	4	6
	Activity as optical density			
1	0.284	0.164	0.090	0.034
2	0.280	0.174	0.080	0.031
3	0.284	0.182	0.093	0.039
4	0.296	0.181	0.103	0.040
5	0.288	0.185	0.101	0.046
Mean	0.287	0.177	0.0935	0.038
S.D.		0.00625	0.00845	0.0058
S.D., *enzyme units*		0.18	0.21	0.25
Coefficient of variation, %		9.0	5.3	4.2

optimum ionic strength, it is the minimum concentration of buffer which will maintain pH when various unknown samples are being analyzed.

Reproducibility—Table VI illustrates a series of experiments in which repeated determinations of a standard enzyme preparation were made. Each determination represents the average of two tubes. Two experiments were run by one operator and three by another. It will be noted that good agreement is obtained at all levels. The standard deviation in terms of units of enzyme was obtained by constructing a curve of the means and determining the variations of the individual experiments at each level.

The standard deviation in terms of optical density closely approximates the accuracy of the Coleman junior spectrophotometer, indicating that further changes in technique are unlikely to increase precision unless some other type of instrument is used.

DISCUSSION

The estimation of hyaluronidase by the viscosity method represents a tedious and time-consuming process. Previous investigators have proposed the use of a turbidity method. These have not gained general favor because of instability of reagents and lack of standardization. Exact conditions for the use of this assay have now been determined. The reagents used are stable and are easily reproducible.

The literature on hyaluronidase is complicated by the various units introduced by different investigators. Since these vary widely with conditions of pH and salt concentration as well as with purity of substrate, it seems unwise at the present time to introduce a new unit. Since hyaluronidase is a relatively stable enzyme, we have used an arbitrary unit based on a given amount of partially purified enzyme. This can be compared with any unknown enzyme by any method of assay.

SUMMARY

1. Conditions for the use of the turbidity method for the determination of testicular hyaluronidase have been studied in detail.

2. A simple, rapid, and accurate procedure has been devised which utilizes stable reagents.

3. It is proposed that pending further knowledge the unit of hyaluronidase be based on a given amount of standard enzyme.

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THE KINETICS OF THE ENZYMATIC HYDROLYSIS OF HYALURONIC ACID

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The wide-spread natural occurrence of the enzyme hyaluronidase has been recognized for a number of years. The recent demonstration of the possible rôle of testicular hyaluronidase in fertilization (1–3), the inhibition ot hyaluronidase by salicylates (4, 5), and the presence of a hyaluronidase inhibitor in human blood have focused attention on this enzyme (6, 7). Relatively little attention has been paid to the kinetics of the reaction catalyzed by hyaluronidase. It is the purpose of this communication to present evidence that, within the usual assay range, this reaction is first order with respect to hyaluronic acid, with changing rate due to changing substrate concentration.

A variety of different methods have been devised for the assay of hyaluronidase. These have been discussed in the accompanying paper (8).

The viscosity method was first studied in detail by Madinaveitia and Quibbel (9). These workers found that the time required for half reduction in viscosity (half life time) is independent of substrate concentration and inversely proportional to enzyme concentration. This has since been confirmed by McClean (10) and Haas (6).

The viscosity reduction method depends on the rate of decrease in the viscosity of a solution of hyaluronic acid. It has been customary to determine the hyaluronidase activity in terms of the reciprocal (or some function thereof) of the half life time.

Recently Swyer and Emmens (11) have reinvestigated the relationship of activity and substrate concentration and have found that at lower concentrations of substrate the activity varies with substrate concentration. These workers have also raised objections to the expression of activity as a reciprocal of the half life time and suggested an alternate procedure which depends on the relationship of the log of concentration of enzyme and the viscosity at a specific time.

When the fall in viscosity is plotted against time, a characteristic curve is obtained. It has been assumed by some that this represents a first order reaction. Thus Werthessen *et al.* (12) have used this assumption in determining the hyaluronidase content of human semen. When one plots log C against t, a straight line is not obtained; thus doubt is cast on the validity of the application of the equation for a first order reaction.

It has seemed to us that this situation might be explained by the fact that the substrate concentration decreases with time, with a consequent decrease in concentration of enzyme-substrate complex. According to the Michaelis-Menten theory the rate of reaction depends on the concentration of enzyme-substrate complex (13).

The relationship between the substrate concentration and the amount of enzyme-substrate complex during any time interval is given by Equation 1.

$$K_s = \frac{(E_a)\ (S_a)}{(ES_a)} \tag{1}$$

K_s = dissociation constant
(E_a) = average concentration of free enzyme from time 0 to time t
(S_a) = average concentration of substrate from time 0 to time t
(ES_a) = average concentration of enzyme-substrate complex from time 0 to time t

$$(E_a) = \Sigma E - ES_a \tag{2}$$

ΣE = total enzyme concentration

Since the velocity constant (k_{v_t}) depends on the enzyme-substrate concentration,

$$k_{v_t} = A(ES_a) \tag{3}$$

$$(ES_a) = \frac{k_{v_t}}{A}$$

$$A = \text{a constant}$$

k_{v_t} = reaction constant for interval from time 0 to time t

$$K_s = \frac{(\Sigma E - ES_a)(S_a)}{(ES_a)} \tag{4}$$

or

$$K_s = \frac{\left(\Sigma E - \frac{k_{v_t}}{A}\right)(S_a)}{\frac{k_{v_t}}{A}} \tag{5}$$

$$K_s = \frac{\left(\frac{A\Sigma E - k_{v_t}}{A}\right)(S_a)}{\frac{k_{v_t}}{A}} \tag{6}$$

$$K_s = \frac{\frac{A\Sigma ES_a - k_{v_t} S_a}{A}}{\frac{k_{v_t}}{A}} \tag{7}$$

(8) $$K_s = \frac{A\Sigma ES_a - k_{v_t} S_a}{k_{v_t}}$$

(9) $$K_s = \frac{A\Sigma ES_a}{k_{v_t}} - S_a$$

$$K_s + S_a = \frac{A\Sigma ES_a}{k_{v_t}}$$

(10) $$k_{v_t} = \frac{A\Sigma ES_a}{K_s + S_a}$$

Equation 10 relates the velocity constant to the average substrate concentration from time 0 to time t, the dissociation constant, a constant A, and the total enzyme concentration. If one now wants to obtain the theoretical velocity constant at time 0, it is related to the constant for any time interval from 0 to t by Equation 11 or 12.

(11) $$\frac{k_{v_0}}{k_{v_t}} = \frac{A\Sigma ES_0}{K_s + S_0} \times \frac{K_s + S_a}{A\Sigma ES_a}$$

or

(12) $$\frac{k_{v_0}}{k_{v_t}} = \frac{S_0}{S_a} \times \frac{K_s + S_a}{K_s + S_0}$$

If we consider the reaction as first order, the velocity constant (k_{v_t}) for a time interval from 0 to t is given by Equation 13.

(13) $$k_{v_t} = \frac{2.303}{t} \log \frac{S_0}{S}$$

S_0 = initial substrate concentration
S = final substrate concentration at time t

It will be convenient then to correct the k_{v_t} at any time t to some standard time. A convenient choice is k_{v_0}, which is the theoretical velocity constant at t_0 with substrate concentration S_0. By combining Equations 12 and 13 we may now write Equation 14 for k_{v_0}.

(14) $$k_{v_0} = \frac{2.303}{t} \log \frac{S_0}{S} \times \frac{S_0}{S_a} \times \frac{K_s + S_a}{K_s + S_0}$$

On inspection of Equation 14 it will be seen that when S_0 (initial substrate concentration) is small compared with K_s the term $(K_s + S_a)/(K_s + S_0)$ approaches unity and k_{v_0} is closely approximated by the expression

(15) $$k_{v_0} = \frac{2.303}{t} \log \frac{S_0}{S} \times \frac{S_0}{S_a}$$

When, however, S_0 is very large compared to K_s, the term $(K_s + S_a)/(K_s + S_0)$ approaches the reciprocal of S_0/S_a and $(S_0/S_a)\ (K_s + S_a)/(K_s + S_0)$ approaches 1 and the standard first order equation holds; *i.e.*,

$$k_{v0} = \frac{2.303}{t} \log \frac{S_0}{S}$$

The experiments presented in this paper were designed to test the validity of these formulations. K_s could not be determined in these experiments, since for technical reasons it is impossible to carry them out in the presence of a great excess of substrate. It has already been shown that the factor $(K_s + S_a)/(K_s + S_0)$ approaches unity at low substrate concentrations, and will therefore be omitted from further considerations.

S_a was obtained graphically from the plot of S against time.

Methods

Materials used were prepared by methods described in the accompanying publication (8). Viscosimetric determinations were carried out essentially according to the method of Haas (6).

The turbidity method used was as described (8) with the exception that samples were withdrawn from the reaction mixture at given time intervals and turbidity was developed and read in the usual way with the Coleman junior spectrophotometer.

EXPERIMENTAL

The first experiments were performed by the viscosimetric method. In order to be able to utilize viscosity data in Equation 15, it had to be shown that under the conditions employed viscosity is directly proportional to hyaluronic acid concentration. The experiment shown in Fig. 1 illustrates that this is the case. Viscosity is expressed as relative viscosity $(\eta - 1)$. This then represents that portion of the relative viscosity which is contributed by the hyaluronic acid.

Fig. 2 illustrates a typical assay in which viscosity is plotted against time. The characteristic curve previously mentioned will be noted. Fig. 3 illustrates the same assay when log S (log of viscosity) is plotted against time. It is readily apparent that the straight line relationship expected in a first order reaction is not obtained. Table I shows the results obtained for k_{v_t} calculated according to Equation 13 (the standard equation for a first order reaction). When, however, k_{v_0} (last column) was calculated according to Equation 15, relatively good constancy was obtained. It should be kept in mind that in addition to the obvious experimental errors (which are particularly large in the first points), these calculations are made on the assumption that the products of the reaction contribute nothing to the viscosity.

Table II illustrates the changes in k_{v_0} with changing enzyme concentrations. It is apparent that k_{v_0} is directly proportional to enzyme concentration except at high enzyme concentrations. Table II also gives the half life time and the enzyme activity as obtained graphically and expressed in the manner of Haas. It is seen that, although this represents a summation of effects due to changing rates, the relationship between concentration and activity is linear in the range of the lower three enzyme concentrations.

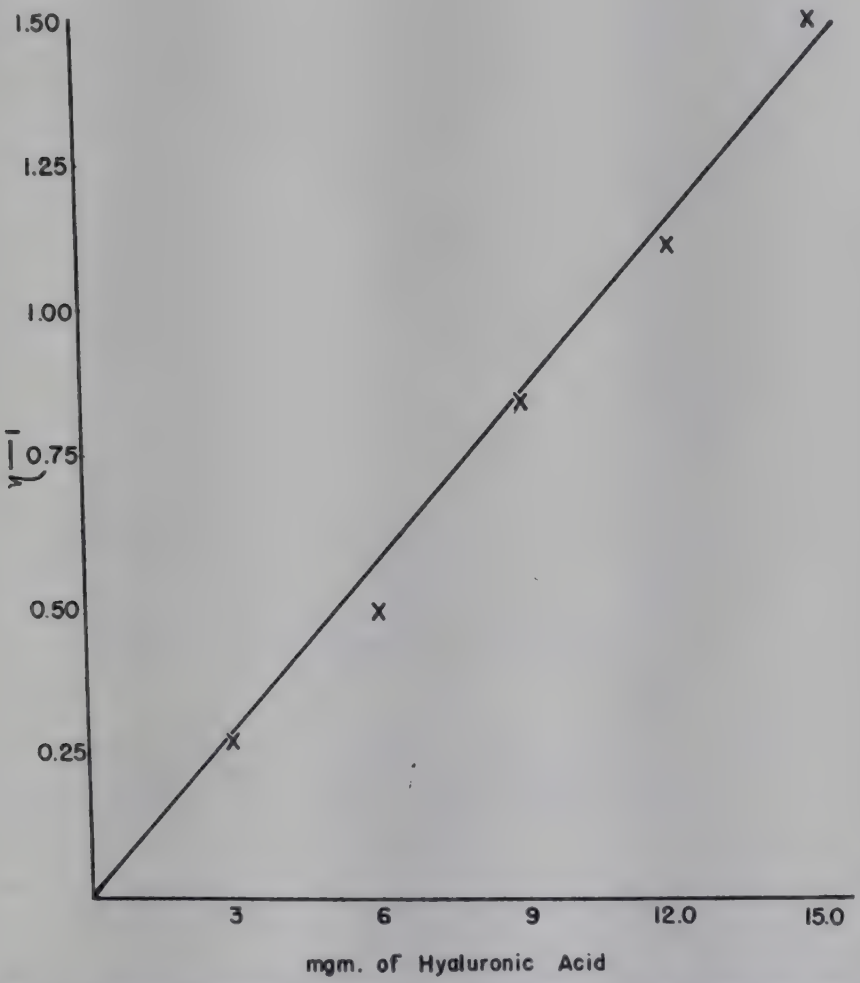

FIG. 1. Relationship of viscosity to hyaluronic acid concentration

The fact that $t\frac{1}{2}$ calculated from k_{v_0} is lower than $t\frac{1}{2}$ obtained graphically again reflects the change in rate with time. The calculated value for $t\frac{1}{2}$ gives the theoretical half life time, if the reaction is proceeding at rate k_{v_0} during the entire course of the reaction.

The next group of experiments was performed by a turbidity method based on the mucin clot phenomenon. An appropriate amount of hyaluronic acid solution mixed with enzyme was incubated at the temperatures indicated.

In order to use this method the relationship between hyaluronic acid concentration and optical density had to be determined. It was found that optical density is directly proportional to hyaluronic acid concentration. These data are presented in the accompanying paper (8).

Fig. 4 represents the change in hyaluronic acid concentration with time. The curve, it will be noted, closely approximates that obtained with the viscosity method (Fig. 2).

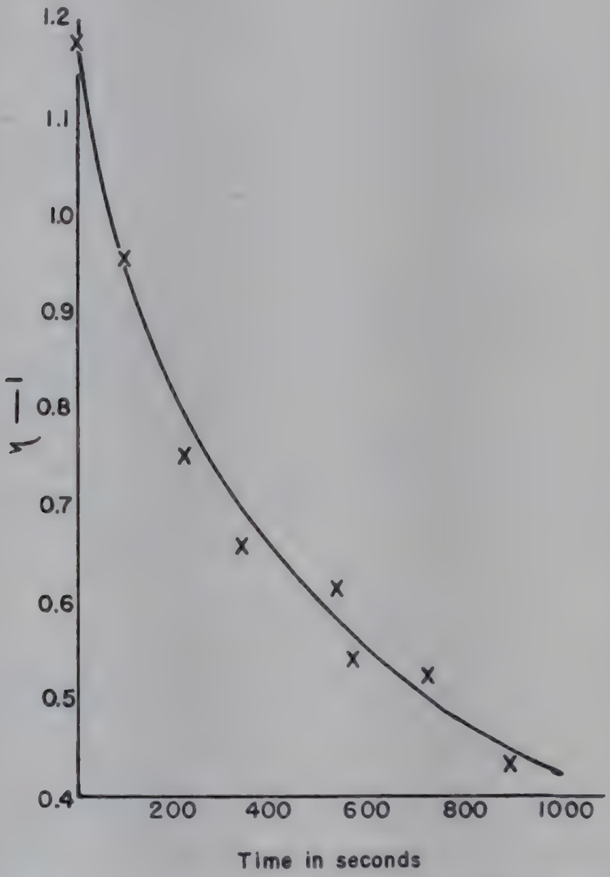

Fig. 2. Change in viscosity plotted against time

The results of the first group of experiments with the turbidity method are illustrated in Table III. The values for k_{v_t} show a trend similar to those obtained with the viscosity method; namely, they drop directly with S_a (average substrate concentration from t_0 to t). When k_{v_0} is calculated by Equation 15, it is noted that good constancy is obtained except during the early periods. This deviation is to be expected in view of the fact that at these times the difference between S_0 and S_a is so small that small errors in the determination of S_a produce very large errors in k_{v_0}.

Table IV shows the average k_{v_0} obtained with various enzyme concentrations. It will be seen that k_{v_0} is directly proportional to enzyme concentration (enzyme concentration is given in arbitrary units). It will be

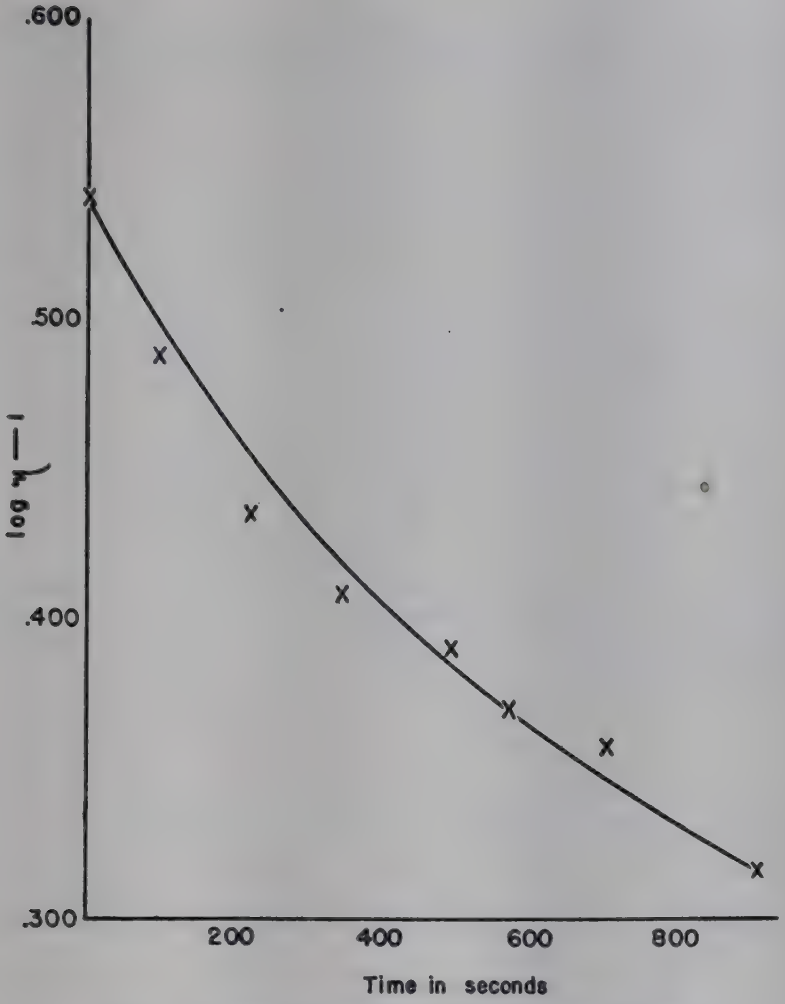

FIG. 3. Log of viscosity plotted against time

TABLE I

k_{v_0} *As Determined by Viscosity Method*

Temperature 38°.

t	S	k_{vt}	S_a	k_{v0}
sec.	$(\eta - 1)$	$\frac{1}{sec.} \times 10^3$	$(\eta - 1)$	$\frac{1}{sec.} \times 10^3$
0	1.18			
95	0.95	2.31	1.05	2.58
212	0.75	2.12	0.94	2.68
336	0.66	1.66	0.86	2.28
490	0.61	1.34	0.80	1.97
586	0.54	1.34	0.76	2.02
674	0.52	1.26	0.70	2.12
876	0.42	1.17	0.65	2.12

noted that $t\frac{1}{2}$ calculated from k_{v_0} bears the same relationship to $t\frac{1}{2}$ obtained graphically as in the viscosity method (Table II).

TABLE II

k_{v_0} at Different Enzyme Concentrations (Viscosity Method)

Temperature 38°.

Concentration of enzyme	Average k_{v0}	$t_{1/2}$*	A†	$t_{1/2}$ (graphic)
units	$\frac{1}{sec.} \times 10^3$	*sec.*	$\frac{1}{sec.} \times 10^3$	*sec.*
1	0.61	1103	0.60	1680
	0.55	1260	0.54	1850
2	0.89	788	0.88	1135
	1.22	570	1.20	830
4	1.80	384	1.96	540
	2.10	330	1.85	510
8	5.15	135	5.10	196
	5.29	131	5.70	176

* Calculated by the formula, $t_{1/2} = 0.693/k_{v_0}$.

† Activity according to Haas.

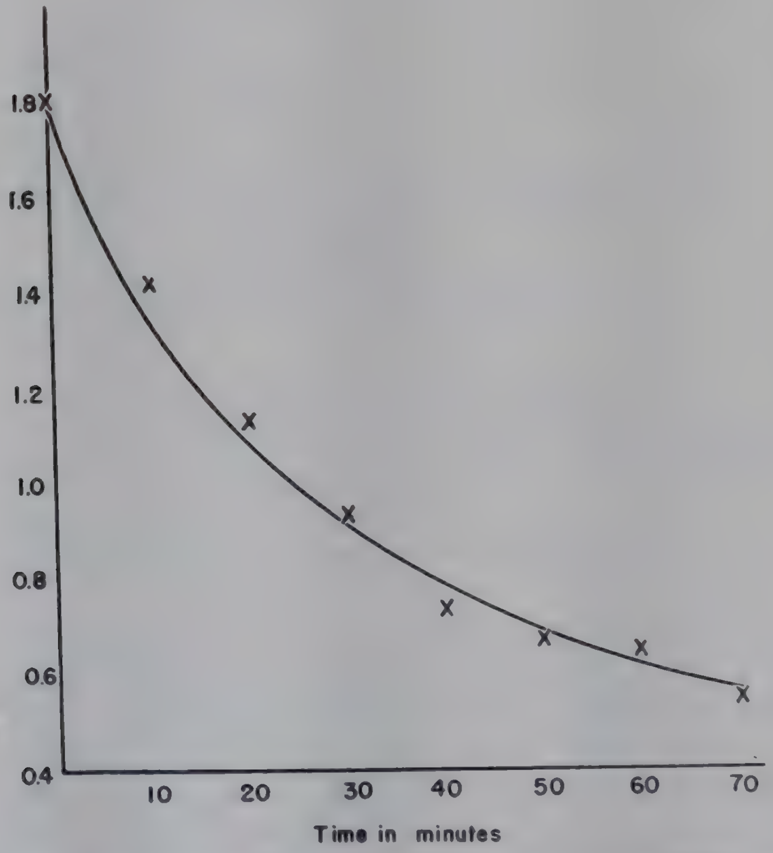

FIG. 1. Change in hyaluronic acid concentration with time (turbidity method)

The next group of experiments was conducted to determine the effect of temperature on the rate of this reaction as measured by k_{v_0}. The only variation in experimental design was the change in temperature. Table V illustrates the results of such experiments, showing that for every 10°

TABLE III

k_{v_0} Determined by Turbidity Method

Temperature 38°.

t	S (hyaluronic acid)	k_{vt}	S_a (hyaluronic acid)	k_{v_0}
sec.	*mg.*	$\frac{1}{sec.} \times 10^4$	*mg.*	$\frac{1}{sec.} \times 10^4$
0	1.80			
600	1.42	3.92	1.62	4.26
1200	1.12	3.96	1.45	4.90
1800	0.94	3.62	1.31	4.95
2400	0.75	3.62	1.20	5.40
3000	0.72	3.06	1.09	5.33
3600	0.63	2.92	1.00	5.26
4200	0.57	2.75	0.93	5.35

TABLE IV

Relationship of k_{v_0} to Enzyme Concentration

Enzyme concentration	k_{v_0}	$t\frac{1}{2}$*	$t\frac{1}{2}$ (graphic)
units	$\frac{1}{sec.} \times 10^4$	*sec.*	*sec.*
2	1.53	4530	6300
4	3.35	2070	2940
6	5.22	1330	1800
8	6.90	1005	1210

* Calculated by the formula, $t\frac{1}{2} = 0.693/k_{v_0}$.

TABLE V

Relationship of k_{v_0} to Temperature

Enzyme concentration	$k_{v_0} = \frac{1}{\text{sec.}} \times 10^4$		
	38°	28°	18°
units			
8	6.9	3.48	
16		7.15	3.84

increase in temperature the reaction rate is doubled. If one calculates the activation energy by the Arrhenius equation from these data, values of 12,800 and 11,000 calories are obtained. This is in the range found for many other enzymatic reactions (14). Previous work by Robertson *et al.* (15) using an enzyme obtained from *Clostridium welchii* showed a K_{10} of 1.75.

DISCUSSION

The Michaelis-Menten theory of enzyme reactions states that the rate of the reaction depends upon the concentration of the enzyme-substrate complex. This in turn depends upon the concentration of the substrate unless the latter is present in excess. Thus as the substrate is used up, the rate of reaction falls, and unless correction is made for this effect, a reaction which is truly first order in nature will apparently not obey the standard equation for such a reaction. The expression derived in this paper makes it possible to correct for this effect.

It has been assumed in this derivation that the reaction is first order in nature with respect to substrate. According to the Michaelis-Menten theory this will be true only when the enzyme is not saturated. When the enzyme is essentially completely saturated, the rate becomes independent of substrate concentration and the reaction behaves as a zero order reaction with respect to substrate. Because of this phenomenon the equation derived cannot be used to compare different initial substrate concentrations. The k_{v_0} derived is not an absolute constant but depends on the particular initial substrate concentration. It is useful in that it is directly proportional to enzyme concentration. This may have a more general application in the study of other systems in which it is inconvenient or impossible to use a large excess of substrate.

SUMMARY

1. The kinetics of the hydrolysis of hyaluronic acid under the influence of hyaluronidase have been studied by two different methods.

2. If the changing substrate concentration is taken into account, the data indicate that this reaction is first order in character under the conditions studied.

3. An expression has been derived for the theoretical reaction velocity constant at 0 time. This has been found to fit the experimental data.

4. The energy of activation calculated by the Arrhenius equation was found to be in the neighborhood of 12,000 calories.

5. The expression derived is of a general nature and should be of importance in the study of any system in which the concentration of substrate is below that necessary for saturation of the enzyme during the course of the reaction.

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STUDIES ON THE CYCLOPHORASE SYSTEM

I. THE COMPLETE OXIDATION OF PYRUVIC ACID TO CARBON DIOXIDE AND WATER

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The present communication is the first in a series devoted to documenting the properties of a complex of enzymes which catalyzes the complete oxidation of pyruvic acid by way of the Krebs citric acid cycle (1, 2) and which has been named the cyclophorase system. The cyclophorase system has been found to be widely distributed in mammalian tissues, with the highest concentration in liver and kidney.

Preparation of Enzyme Complex—Rabbit kidney provides a rich and convenient source for preparation of the cyclophorase system. The full details of the preparation are given in the experimental section. The method consists essentially in mincing fresh kidney in 0.9 per cent potassium chloride at 0° and adding alkali in an amount just sufficient to neutralize the acid formed when the cells are ruptured.[1] The easily sedimentable fraction of the resulting homogenate contains the cyclophorase system, and this fraction is purified by repeated suspension in potassium chloride solution followed by centrifugation. The activity of different batches of "enzyme" is remarkably constant (Q_{O_2} about 30). In several hundred runs by the above method no inactive preparations were obtained. The necessity for neutralizing the acidity which develops during mincing has been established only in the case of the preparation from rabbit kidney. It does not apply to the preparations from cat heart or rat liver. This would suggest that in rabbit kidney certain destructive enzymes are prevented from inactivating the cyclophorase system when the pH is maintained above 7.0, whereas in other tissues such as liver and heart these destructive enzymes either are not present or are present in much smaller concentration. Consistent with this interpretation is the fact that the well washed cyclophorase preparation of rabbit kidney, *e.g.* at the Residue R_3[2] stage, is stable and active at the same pH (about 6.0) at which the cyclophorase system becomes inactivated in the original homogenate.

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* National Research Council Fellow in the Medical Sciences.

[1] Microscopic examination has shown practically no intact cells in the homogenate, though the cell nuclei are still essentially undamaged.

[2] The sediment obtained by centrifuging the homogenate will be referred to as the first residue or Residue R_1. The residue obtained by resuspending Residue R_1 in salt solution and then centrifuging will be referred to as Residue R_2, etc. The organ from which the enzyme is prepared is indicated by the other initial, in capital letter, thus Residue R_2K for the second residue from kidney.

The preparation of the cyclophorase system from rabbit kidney is active over a period of 12 hours when stored at 0°. In rare cases activity has been found after 4 days but, in general, less than half the original activity survives the 1st day. The enzyme system does not tolerate freezing or drying, exposure to high concentrations of salt (greater than 0.1 M), to solvents (greater than 10 per cent by volume), to deionized water, to acidity below that of pH 4.0, or to alkalinity above that of pH 10.

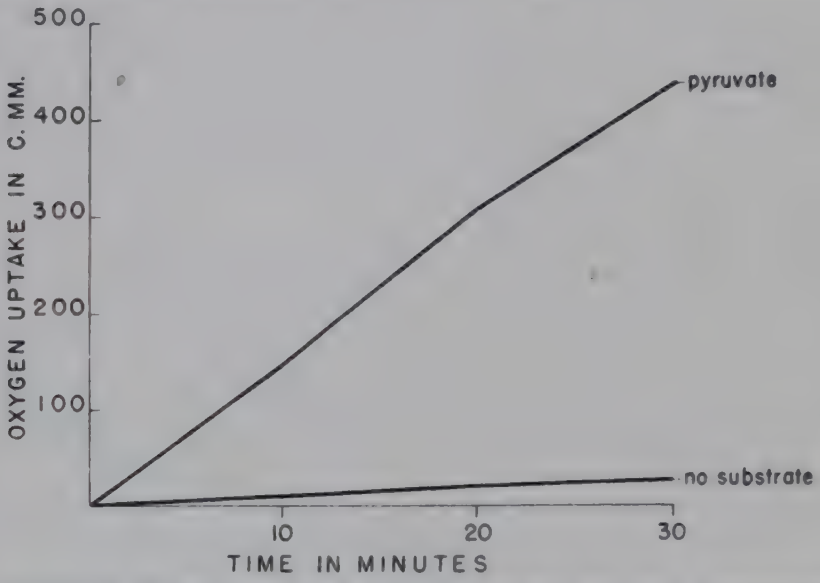

FIG. 1. Oxidation of pyruvate by a freshly prepared enzyme preparation. Each manometer vessel contained 1.5 cc. of Residue R_3K, 0.2 cc. of 0.125 M phosphate buffer of pH 7.3, and 0.2 cc. of 0.1 M $NaHCO_3$. In the experimental cup, 0.5 cc. of 0.1 M lithium pyruvate was added. The final volume was made up to 3 cc. with water; alkali in the center well, oxygen in the gas space; bath maintained at 38° and 5 minutes equilibration in the bath before closing the taps.

The enzyme suspension is a highly viscous, faintly yellow-colored gel. Procedures or conditions which lead to inactivation of the cyclophorase system in many cases lead to visible alteration in the appearance of the gel from its characteristic smooth consistency to one that resembles curdled milk. Elsewhere evidence will be presented that the enzyme preparation from rabbit kidney or liver is largely nucleoprotein in nature, though the cyclophorase system has yet to be identified with the nucleoprotein in the preparation. There are not enough data to decide whether the enzymes constituting the cyclophorase complex are just a loose association of chemically and physically discrete enzymes or whether they are all inextricably associated with one another as in a mosaic.

Components of Cyclophorase System—When freshly prepared, the enzyme system requires only molecular oxygen for the oxidation of pyruvic acid (*cf*. Fig. 1). With storage at 0° the enzyme preparation undergoes some change by virtue of which it cannot oxidize pyruvate unless supplemented with adenosine triphosphate (or adenylic acid) and magnesium ions (*cf.* Fig. 2). Not all the oxidizing enzymes involved in the over-all oxidation of pyruvic acid require these two components. Elsewhere the requirements of the individual oxidizing enzymes for various components will be considered in detail.

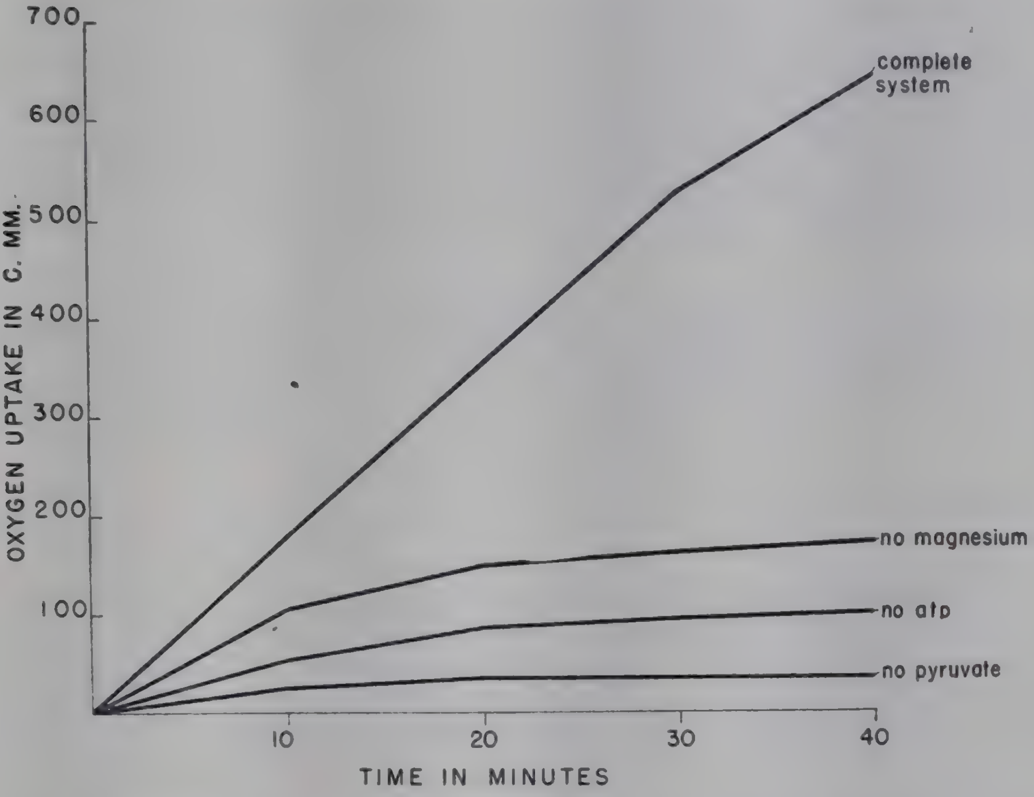

FIG. 2. Components required for the oxidation of pyruvate by an aged enzyme preparation. The complete system contained 1 cc. of a 4 hour-old Residue R_3K, 0.5 cc. of 0.1 M pyruvate, 0.2 cc. of 0.02 M magnesium sulfate, 0.3 cc. of 0.01 M adenosine triphosphate (*atp*), 0.5 cc. of 0.1 M sodium bicarbonate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.3, alkali in the center well (0.2 cc.), and water to make up to a final volume of 3 cc. Oxygen in the gas space.

Individual Steps in Complete Oxidation of Pyruvic Acid

Krebs and his colleagues in their now classical investigations (1, 2) established that pyruvic acid is oxidized to completion by way of the so called citric acid cycle. They employed in their experiments homogenates of pigeon breast muscle and of other tissues. The experiments reported below establish in a more precisely defined enzyme system, *viz.* the cyclophorase system of rabbit kidney, the same series of reactions demonstrated by Krebs in studies with homogenates and indicate the cyclophorase system as the one which implements the reactions of the citric acid cycle.

Since the oxidation of pyruvic acid is cyclical in nature, it does not matter which reaction will be the point of departure. For convenience, the oxidation of α-ketoglutarate to succinate will be considered first.

Oxidation of α-Ketoglutarate—In presence of 0.033 M malonate, the oxidation of α-ketoglutarate by the kidney enzyme system only proceeds one step to succinate, in agreement with the results of Ochoa (3) in his study of an analogous enzyme prepared from cat heart.

$$\text{(1)}\quad COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH + \tfrac{1}{2}O_2 \rightarrow COOH\cdot CH_2\cdot CH_2\cdot COOH + CO_2$$

The supporting data are shown in Table I. Succinic acid was isolated from the reaction mixture and characterized by melting point (182° before and after admixture with a known sample) according to the procedure described by Stumpf *et al.* (5) for separating succinic acid from malonic acid and α-ketoglutaric acid.

Table I

Oxidation of α-Ketoglutarate to Succinate and Carbon Dioxide in Presence of Malonate

	α-Ketoglutarate disappearing	Oxygen taken up	Carbon dioxide formed
	micromoles	*microatoms*	*micromoles*
Observed	28.0	27.1	27.0
Theory		28.0	28.0

The following additions were made in the manometer cup: 1 cc. of Residue R_3K, 0.1 cc. of M malonate, 0.3 cc. of 0.1 M α-ketoglutarate, 0.5 cc. of 0.04 M phosphate buffer of pH 7.3, and 0.2 cc. of 0.02 M magnesium sulfate. Alkali in the center well and oxygen in the gas space. Time of experiment, 90 minutes at 38°. α-Ketoglutaric acid was estimated by the bisulfite method of Clift and Cook (4). The values in the table have all been corrected for the small blanks without added α-ketoglutarate.

Oxidation of Succinate—The one-step oxidation of succinate to fumarate can be isolated for study by the expedient of shaking the enzyme preparation at 38° for some 15 minutes before tipping in the succinate. This treatment completely destroys the other oxidizing enzymes and thus makes it possible to study exclusively the action of succinoxidase.

$$\text{(2)}\quad COOH\cdot CH_2\cdot CH_2\cdot COOH + \tfrac{1}{2}O_2 \rightarrow COOH\cdot CH{=}CH\cdot COOH \rightleftharpoons COOH\cdot CHOH\cdot CH_2\cdot COOH$$

The product of oxidation, *viz.* fumarate, is brought into equilibrium with *l*-malic acid under the influence of fumarase which is present in the cyclophorase preparation. The data in Table II show that the requirements of equation (2) are satisfied.

The oxidation of succinate beyond the fumarate-malate stage can also be arrested with 0.0033 M arsenite, a reagent which Krebs used successfully for the same purpose in his experiments with homogenates of pigeon breast muscle.

TABLE II

Oxidation of Succinate to Fumarate

	Succinate oxidized	Oxygen absorbed	Malate formed	Malate + fumarate*
	micromoles	*micromoles*	*micromoles*	*micromoles*
Observed......	50.0	49.6	44	50
Theory........		50.0	44†	50

The experimental vessels contained 1 cc. of Residue R_3K, 0.5 cc. of 0.1 M succinate (in the side arm), 0.2 cc. of 0.125 M phosphate buffer of pH 7.3, and water to a final volume of 3 cc.; alkali in the center well and oxygen in the gas space. After the manometer vessel was shaken in the bath (38°) for 15 minutes, the taps were closed and succinate was tipped in from the side arm. All of the experimental values have been corrected for the appropriate blanks. Each of the observed values represents the mean of at least two estimations. Agreement was within 3 per cent. Malic acid was estimated by the method of Pucher *et al.* (6).

* The equilibrium ratio of malate to fumarate has been found to be 7.66 under the same conditions as in the oxidation of succinate.

† The "theoretical" value of 44 micromoles is the experimental value obtained after adding 50 micromoles of malic or fumaric acid to the enzyme mixture.

TABLE III

Oxidation of Malate and Fumarate to Pyruvate

	Oxygen absorbed	Carbon dioxide formed	Pyruvic acid formed
	microatoms	*micromoles*	*micromoles*
Malate, observed........	14.0	13.0	12.4
" theory...........		14.0	14.0
Fumarate, observed......	13.4	13.4	13.5
" theory........		13.4	13.4

Each experimental manometer vessel contained 1.5 cc. of Residue R_3K, 0.5 cc. of 0.2 M 2-amino-2-methyl-1,3-propanediol buffer of pH 9.5, 0.2 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.1 cc. of 0.125 M phosphate buffer of pH 7.2, 0.15 cc. of 10 N sulfuric acid in the center well, and 0.2 cc. of 0.1 M *l*-malate or fumarate. After 15 minutes equilibration in the bath, the taps were closed. The oxygen uptake for this period with the taps open was obtained by extrapolation. At the end of the run (70 minutes) acid was tipped in from the center well and the total carbon dioxide formation was determined. Pyruvic acid was determined by the colorimetric procedure of Straub (7). A known amount of pyruvic acid (10 and 20 micromoles) was added to the enzyme mixture as above, but without malate or fumarate, and run through as standards with which to compare the experimental solutions. All estimations were carried out in duplicate and the values recorded above are the mean values, agreement being within 5 per cent in all cases.

Oxidation of l-Malate—At pH 9 the oxidation of *l*-malate proceeds only one step, with accumulation of pyruvate (*cf.* Table III). The analytical data satisfy the requirements of equation (3).

$$COOH \cdot CHOH \cdot CH_2 \cdot COOH + \tfrac{1}{2}O_2 \rightarrow CH_3 \cdot CO \cdot COOH + CO_2 \tag{3}$$

Oxalacetate is, in fact, the first product of oxidation. However, it cannot be demonstrated as an intermediary under these conditions. When added to the enzyme preparation, it is broken down to pyruvate and carbon dioxide at a sufficiently rapid rate to preclude any chance of its being isolated without employing some trapping device. The decarboxylation of oxalacetic acid is partly catalyzed and partly spontaneous in the pH range 7 to 9. No additions in the way of cations, such as Mg or Mn, are needed for the full activity of oxalacetic carboxylase.

With the aid of cyanide, it becomes possible to demonstrate that oxalacetic acid is the first product of oxidation of *l*-malate. Since cyanide inhibits the system involved in the reaction with molecular oxygen, methylene blue has to be added in order to make possible the reaction with molecular oxygen (*cf.* Table IV). Whereas 1 mole of CO_2 is formed for each atom of oxygen taken up during the oxidation of *l*-malate at pH 9, no CO_2 is formed when the oxidation takes place in presence of cyanide. Oxalacetic acids reacts almost instantaneously with cyanide to form the cyanohydrin (9) and no free keto acid can be demonstrated. However, the cyanohydrin can be decomposed in strongly alkaline solution and the presence of a keto acid is now demonstrable.

Pyruvic acid was demonstrated as the product of oxidation of *l*-malate by isolation from the reaction mixture as the 2,4-dinitrophenylhydrazone. After recrystallization from ethyl acetate-ligroin mixtures, the derivative melted at 212°. Analysis showed 40.55 per cent C, 3.20 per cent H, and 20.61 per cent N; theory for $C_9H_8O_6N_4$ requires 40.28 per cent C, 3.01 per cent H, and 20.90 per cent N. The isolation was carried out in a reaction mixture buffered at pH 9.5, the pH at which pyruvic acid largely accumulates.

Conversion of Oxalacetate to Citrate—In presence of barium ions and oxygen, the kidney preparation catalyzes the oxidative conversion of oxalacetic acid to citric acid (*cf.* Table V[3]). Wieland and Rosenthal (10) were the first to use barium ions as a device for accumulating citric acid. No other additions are necessary for citric acid formation from oxalacetate whether the kidney "enzyme" used is freshly prepared or some hours old. The yield of citric acid calculated on the basis of the original starting amount of oxalacetic acid is admittedly low. However, it can readily be shown that the bulk of the oxalacetic acid which disappears either undergoes dismutation and oxidation-reduction, resulting in the formation of malate, fumarate, and succinate (*cf.* Table VI), or accumulates as pyruvic acid. As yet, no means have been found whereby the conversion of oxalacetic to citric acid can be studied without these other side reactions taking place.

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[3] We are much indebted to Mr. Edward Sperling for carrying out many of these estimations.

Pyruvate is barely, if at all, oxidized by a well washed kidney enzyme such as Residue R_6 except in presence of CO_2 or in presence of catalytic amounts of a member of the citric acid cycle (*cf.* Fig. 3). These observations are consistent with the assumption that pyruvic acid is carboxylated to form oxalacetic acid and they confirm the results of Wood *et al.* (11) and Evans *et al.* (12) obtained with the use of isotopes on the reversibility of the decarboxylation of oxalacetic acid in animal tissues. On purely chemical grounds it appears more likely that a condensation between pyruvic acid and oxalacetic acid then takes place rather than between 2 molecules of oxalacetic acid. Elsewhere evidence will be presented that the kidney enzyme preparation catalyzes the condensation of oxalacetic acid with

TABLE IV

Oxidation of Malate and Fumarate to Oxalacetate

No carbon dioxide or pyruvic acid was formed.

	Oxygen absorbed	Keto acid estimated after exposure to alkali
	microatoms	*micromoles*
Malate	10.7	12.3
Fumarate	8.9	7.9

The experimental manometer vessels contained 1.5 cc. of Residue R_3K, 0.05 cc. of 1 M fumarate or malate, 0.5 cc. of 0.1 per cent methylene blue, 0.2 cc. of 0.05 M NaCN (acidified to pH 9) in the side arm, and 0.15 cc. of 10 N sulfuric acid in the center well. The final volume was made up to 3 cc. with water. After the manometers were shaken for 5 minutes in the bath, the taps were closed and the cyanide solution tipped in from the side arm. The oxygen uptake was then followed until complete (20 minutes). Then the acid was tipped in from the center well and the total carbon dioxide formed in the reaction was measured. Two separate types of controls were run: one, the usual counterpart to the experimental except that no substrate was added; the other, identical with the experimental except that strong acid was tipped in at zero time (closing of taps) before the cyanide. This control provides a measure of the carbon dioxide formed during the 5 minute equilibration before the taps were closed and before the oxidation of malate or fumarate was stopped at the oxalacetate stage by cyanide. Pyruvic acid was estimated in the trichloroacetic acid filtrates by the method of Straub (7). The cyanohydrin was decomposed as follows: 2 cc. of the trichloroacetic acid filtrate were mixed with 2 cc. of N alkali. After 5 minutes, the alkaline extract was quickly poured into 5 cc. of 0.5 per cent 2,4-dinitrophenylhydrazine solution in 4 N HCl. Keto acid was then estimated by the procedure of Friedemann and Haugen (8). As standard, known amounts of pyruvic acid or oxalacetic acid were added to the enzyme mixture as above except that substrate was omitted, and these standard solutions were then treated exactly as the experimental solutions. The decomposition of cyanohydrin is not a quantitative procedure and only some 50 per cent of the keto acid originally added could be recovered. When the alkali treatment was omitted, no keto acid could be detected in the trichloroacetic acid filtrate.

other partners such as acetoacetic acid. The data of Tables V and VI, as well as of Fig. 3, are consistent with equation (4), although they do not establish its validity.

TABLE V

Formation of Citrate from Oxalacetate

	Citric acid formed
	micromoles
Complete system	3.5
Without barium	1.9
" oxalacetate	0
" oxygen	1.7
Complete system but with boiled enzyme	0.1

The yield of citric acid in this experiment was 3.5 × 2 × 1.3 × 1/50 × 100 = 18.2 per cent. The factor 1.3 corrects for the amount of *cis*-aconitic and isocitric acids present in equilibrium with citric acid. The complete system contained 1.5 cc. of Residue R_3K, 0.1 cc. of M barium chloride, 0.5 cc. of 0.1 M oxalacetate, and water to a final volume of 3 cc. Oxygen in the gas space; time of experiment, 60 minutes.

TABLE VI

Carbon Balance Study of Anaerobic Disappearance of Oxalacetate

	Formed	Disappearing
	micromoles	*micromoles*
Citrate	4.6	
Pyruvate	21	
Malate + fumarate	17	
Succinate	5.0	
Oxalacetate		50

The recovery of oxalacetate in the form of these four compounds is 104 per cent, if we allow for the fact that 1 molecule of citrate is equivalent to 2 molecules of oxalacetate and that for each molecule of pyruvic acid and citric acid formed 2 and 1 molecules respectively of carbon dioxide are evolved. The experiments were set up in Thunberg tubes. The experimental tubes contained 2 cc. of Residue of R_3K, 0.25 cc. of 0.125 M phosphate buffer of pH 7.3, 0.5 cc. of 0.1 M oxalacetate, and 0.25 cc. of water. The blank tubes contained no substrate. After evacuation the tubes were kept at 38° for 1 hour. Depending upon the estimation, the contents of each set of experimental and control tubes were deproteinized either with trichloroacetic acid or with sulfuric-tungstic acid. For the methods of estimation see the experimental section. Each estimation was carried out at least in duplicate.

$$\text{(4)} \qquad \text{COOH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH} + \text{CH}_3\cdot\text{CO}\cdot\text{COOH} \xrightarrow{\frac{1}{2}\text{O}_2} \text{citric acid} + \text{CO}_2$$

The rabbit kidney enzyme system contains the enzyme, discovered by Martius (13), which brings isocitric, citric, and *cis*-aconitic acids into equilibrium with one another (*cf*. Table VII). Thus, citric acid, when referred to in connection with the kidney system, must be considered to be accompanied by an equilibrium amount of isocitric and *cis*-aconitic acids. It

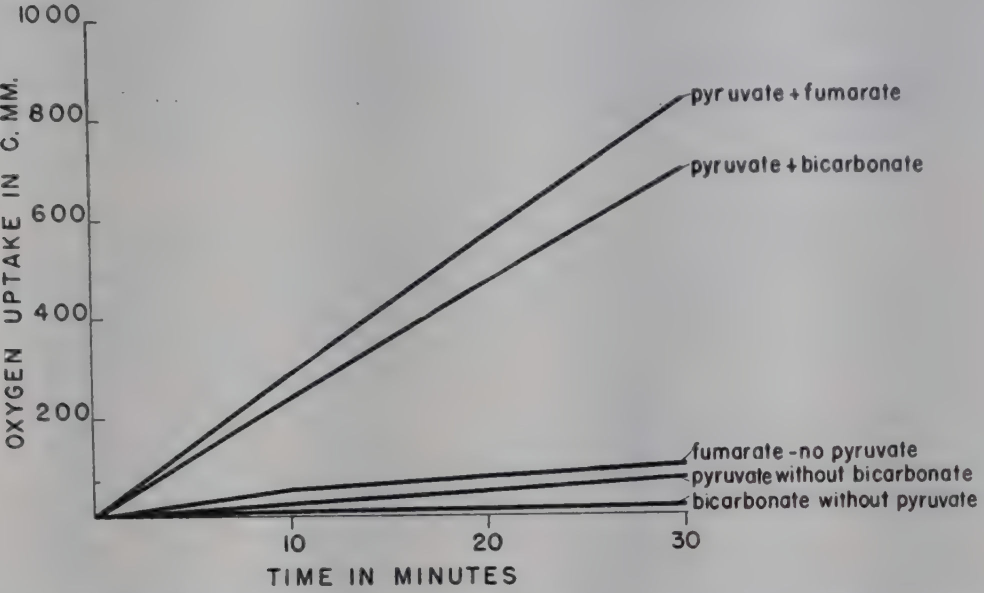

FIG. 3. Sparking of pyruvate oxidation with bicarbonate or with fumarate in catalytic concentration. The complete systems contained 1 cc. of Residue R_3K, 0.5 cc. of 0.1 M pyruvate, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M $MgSO_4$, 0.1 cc. of 0.125 M phosphate buffer of pH 7.3, and either 0.5 cc. of 0.1 M bicarbonate or 0.03 cc. of 0.1 M fumarate.

TABLE VII

Equilibrium of Citric Acid with Isocitric and cis-Aconitic Acids

Addition	Citric acid found at equilibrium
	micromoles
60 micromoles citric acid	47
60 " isocitric acid	46
60 " *cis*-aconitic acid	46
60 " *trans*-aconitic acid	0.2
None	0

The experiments were carried out anaerobically in Thunberg tubes for 45 minutes at 38°. The additions were 1.5 cc. of Residue R_3K, 0.2 cc. of 0.125 M phosphate buffer of pH 7.2, and 0.6 cc. of 0.1 M substrate. The final volume was made up to 3 cc. with water. Citric acid was estimated essentially by the method of Pucher *et al.* (6, 14).

should also be stressed that there is no evidence as yet bearing on the question which of the three equilibrium forms is the actual product of the oxidative condensation. Citric acid happens to be the form actually estimated, but otherwise there is no reason for selecting citric acid rather than the other two forms.

The properties of citrogenase, the enzyme complex which catalyzes the formation of citric acid from oxalacetic acid, has been studied in some detail by Breusch (15, 16), Wieland and Rosenthal (10), and Hunter and Leloir (17). There is still uncertainty as to the mechanism and details of both the condensation and oxidation reaction involved in citric acid formation.

TABLE VIII

Oxidation of Citrate and Isocitrate to α-Ketoglutarate

Substrate	Concentration of arsenite	Oxygen absorbed	α-Ketoglutarate formed
	M	*micromatoms*	*micromoles*
Citrate	0.0066	7.8	5.6
"	0.0066	7.4	6.3
"	0.0066	9.1	7.6
"	0.0033	12.1	9.6
Isocitrate	0.0066	5.0	4.2
"	0.0033	7.9	7.5

The experimental manometer flasks contained 1.5 cc. of Residue R_3K, 0.4 cc. of 0.1 M citrate or isocitrate, arsenite in a final concentration of either 0.0066 or 0.0033 M, alkali in the center well, and water to a final volume of 3 cc. The experiments were carried out for 30 to 60 minutes at 38° with oxygen in the gas space. The values for the oxygen uptake and formation of α-ketoglutarate have been corrected for their appropriate blanks. An internal standard was used in the estimation of α-ketoglutarate by the method of Friedemann and Haugen (8). An enzyme mixture as above was prepared containing 10 micromoles of α-ketoglutarate but no citrate or isocitrate. This mixture was then carried through the procedure, identical to that of the experimental solution. The oxygen uptake in the cup containing α-ketoglutarate was taken as the appropriate blank value for correcting the rates obtaining in presence of citrate or isocitrate.

Oxidation of Isocitrate—At pH 9, the oxidation of isocitrate (or citrate and *cis*-aconitate) to α-ketoglutarate proceeds according to equation (5):

$$\text{COOH}\cdot\text{CH}\cdot(\text{CH}_2\cdot\text{COOH})\cdot\text{CHOH}\cdot\text{COOH} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{COOH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH} + \text{CO}_2 \tag{5}$$

Table VIII shows that the analytical data satisfy the requirements of equation (5). α-Ketoglutaric acid was isolated from the reaction mixture as its 2,4-dinitrophenylhydrazone. After three recrystallizations from aqueous alcohol, the derivative was found to contain 40.9 per cent C and 3.00 per cent H; theory for $C_{11}H_{10}O_8N_4$, 40.5 per cent C and 3.07 per cent H.

Ochoa (18) has shown that a soluble pyridinoprotein enzyme of heart muscle catalyzes the oxidation of isocitrate to oxalosuccinate, which in turn is decarboxylated to α-ketoglutarate and carbon dioxide. Oxalosuccinate may well be an intermediary in the oxidation of isocitrate in the cyclophorase system, but as yet no direct evidence can be brought to bear. Under the conditions of our experiments, oxalosuccinate is broken down so quickly to α-ketoglutarate that it is virtually equivalent to adding α-ketoglutarate. There is no evidence of a carboxylase in the cyclophorase system specific for oxalosuccinate. Apparently the observed breakdown in presence of the enzyme can be accounted for in terms of the spontaneous non-enzymatic reaction.

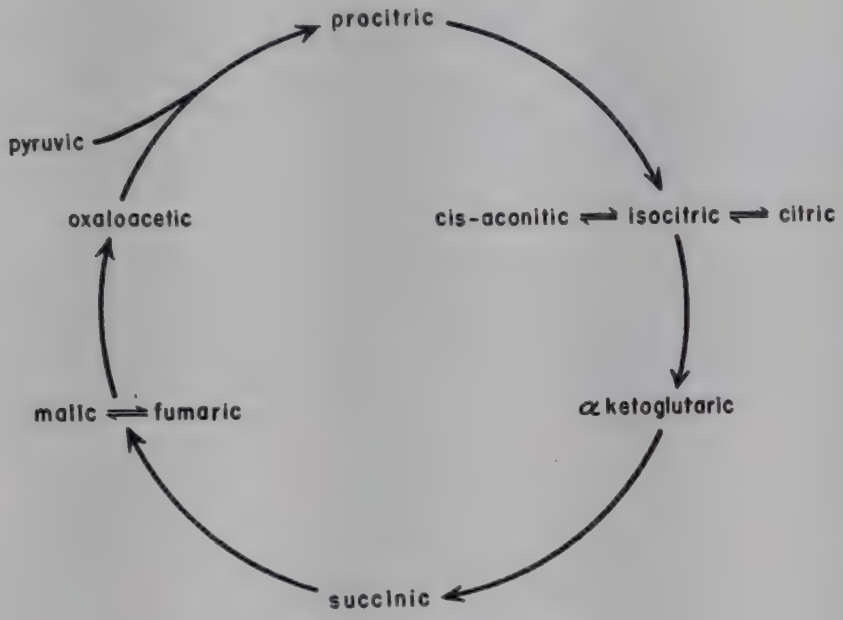

FIG. 4. Citric acid cycle

There is but one indirect bit of evidence which favors oxalosuccinate as an intermediary in the oxidation of isocitrate. α-Ketoglutarate can be reductively carboxylated to citric acid under certain conditions, a process which would argue oxalosuccinate as the primary carboxylation product.

Complete Oxidation of Pyruvate

It follows from what has been said previously that any member of the citric acid cycle (graphically represented in Fig. 4) should be oxidized to carbon dioxide and water in absence of any inhibitors and at pH 7.3. There are only five oxidative steps in one complete turn of the cycle, which means that more than one turn will be needed to oxidize any member of the citric acid cycle that requires per molecule more than 5 atoms of oxygen. In Table IX, evidence is presented[4] that all the postulated members of the cycle are oxidized to completion within the limits of error of the manometric methods. It should also be pointed out that the rates with which all these substances are oxidized are sufficiently rapid to permit the assumption that they share a common pathway of metabolism.

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[4] We are much indebted to Dr. William Atchley for carrying out these manometric experiments.

In the rabbit kidney system, pyruvic acid is oxidized exclusively by way of the citric acid cycle. The system in animal tissues described by Stumpf *et al.* (5), which oxidizes pyruvic acid to acetic acid, apparently is not present in the kidney cyclophorase preparation.

TABLE IX

Complete Oxidation of Members of Citric Acid Cycle

Substrate	Substrate added	Oxygen	
		Observed	Theory for complete oxidation
	micromoles	*microatoms*	*microatoms*
α-Ketoglutarate	5	38.1	40.0
Malate	5	30.7	30.0
"	10	56.8	60.6
Citrate	5	47.1	45.0
Isocitrate	5	45.3	45.0
Succinate	5	34.3	35.0
Fumarate	5	34.0	30.0
Pyruvate	5	23.9	25.0
cis-Aconitate	5	48.4	45.0
Oxalacetate	2.5	12.2	12.5
	Substrate oxidized	Carbon dioxide	
	micromoles	*micromoles*	*micromoles*
α-Ketoglutarate	4.37	21.3	21.9
Isocitrate	3.79	21.7	22.9
Succinate	3.77	15.1	17.2
Fumarate	4.73	16.4	18.9
cis-Aconitate	5.00	27.9	30.0

The complete system in every case contained 1.5 cc. of Residue R_3K, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.2, and substrate in the amount indicated. In the main compartment of both the experimental and blank manometer vessels, 1 micromole of succinate was added to prevent the deterioration of the enzyme during the equilibration period. After 5 minutes, the taps were closed and the substrate solution in the side arm was then mixed with the cup contents. Carbon dioxide formation was measured by the indirect method described fully in Umbreit *et al.* (19). The manometric experiments quoted above are representative experiments which have been consistently duplicated.

Anaerobic Oxidation-Reduction—Oxygen is not an obligatory oxidizing agent for the cyclophorase system. Ferricyanide, for example, can substitute for oxygen effectively, as is shown in Fig. 5. Methylene blue is another satisfactory acceptor. In addition, it is possible to use the oxidant of one

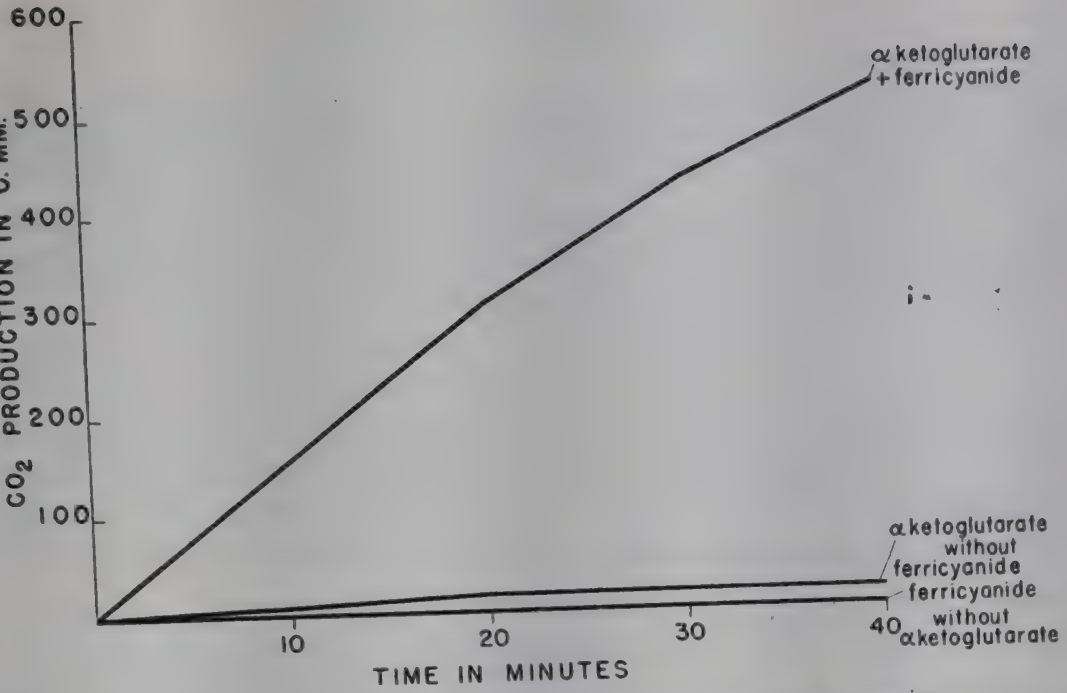

FIG. 5. Oxidation of α-ketoglutarate by ferricyanide. The experimental cup contained 1 cc. of Residue R_3K, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.3 cc. of 0.1 M α-ketoglutarate, 0.1 cc. of 0.5 M sodium bicarbonate, and 0.2 cc. of 0.5 M ferricyanide. The final volume was made up to 3 cc. with water; 95 per cent nitrogen-5 per cent carbon dioxide gas mixture in the gas space.

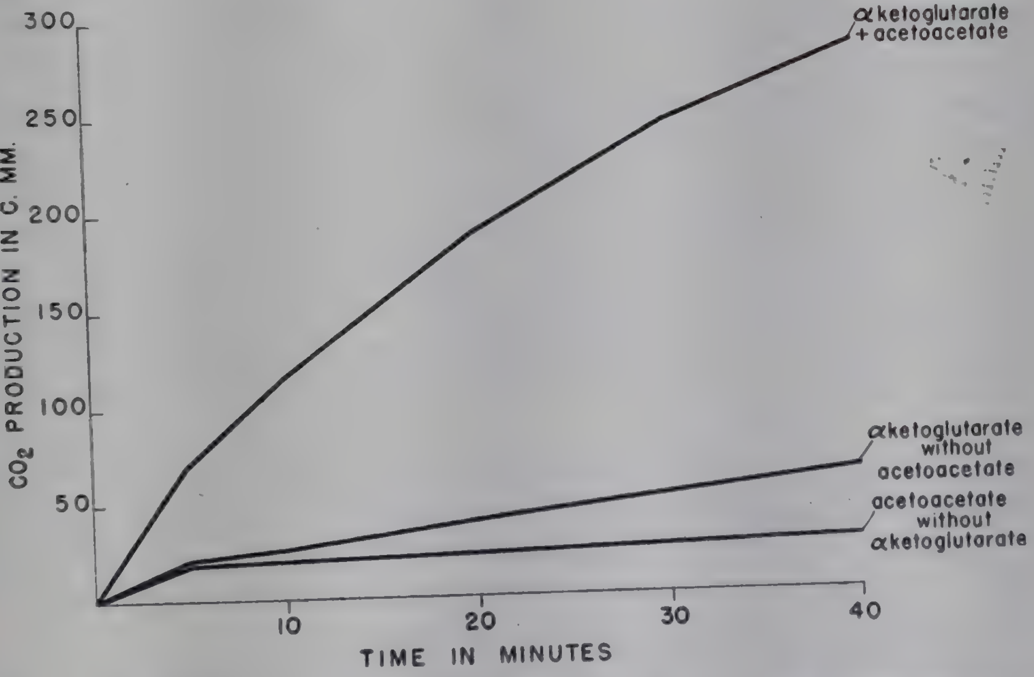

FIG. 6. Oxidation of α-ketoglutarate by acetoacetate. The experimental cup contained 1.5 cc. of Residue R_3K, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.2, 0.3 cc. of 0.1 M α-ketoglutarate, 0.1 cc. of 0.066 M acetoacetate, 0.1 cc. of 0.5 M $NaHCO_3$, 0.03 cc. of M fluoride, and 0.37 cc. of water; 95 per cent nitrogen-5 per cent carbon dioxide gas mixture in the gas space.

oxidation system to oxidize the reductant of another system. Thus, α-ketoglutaric acid can be oxidized by acetoacetate. These particular oxidation reactions can be followed manometrically by measuring carbon dioxide evolution in a bicarbonate medium (*cf.* Fig. 6). A further example of these oxidation-reductions is the oxidation of citrate by various hydrogen acceptors such as acetoacetate and fumarate.

Distribution of Cyclophorase System—Active preparations of the cyclophorase system have been obtained from kidney (rabbit, cat, pigeon, goat, dog, lamb, guinea pig), liver (rabbit, rat, pigeon), heart (cat, pigeon, rabbit), brain (rabbit, pigeon), and breast muscle (pigeon). The kidneys invariably provide rich sources of the cyclophorase system. Liver is also a rich source, but the preparation of active enzyme is more difficult from this tissue.

EXPERIMENTAL

Preparation of Enzyme Complex—Three rabbits (2 to 3 kilos in weight) are killed by a blow on the head and exsanguinated. The kidneys are removed and cooled immediately in ice water. Within 5 minutes the kidneys are blended with 350 cc. of ice-cold 0.9 per cent potassium chloride solution for a period of 2 minutes. Approximately 1.3 cc. of N sodium hydroxide have to be added to maintain the pH between 7 and 7.5 during the blending. The pH should be checked continually by removing drops and testing with brom-thymol blue. The homogenate is centrifuged for 15 minutes in the 1 liter conical head of an International Equipment Company refrigerated centrifuge at 2000 × *g*. The temperature must never be allowed to exceed 5° throughout all manipulations. The supernatant fluid is poured off and replaced by an equal volume of cold potassium chloride solution. The residue (R_1) is evenly resuspended and the mixture centrifuged for 7 minutes. At Residue R_3 stage, the residue is taken up in the minimum volume of cold potassium chloride and a smooth gel is prepared. From the kidneys of three rabbits, approximately 45 cc. of suspension are obtained.

Reagents—The following methods of preparation were used: lithium pyruvate (20), oxalacetic acid (21), α-ketoglutaric acid (22), adenosine triphosphate (19), and *cis*-aconitic acid (23). We are indebted to Dr. Pucher for a sample of the methyl ester of isocitric acid and to Dr. Severo Ochoa for the barium salt of oxalosuccinic acid.

Method of Estimation—The following analytical procedures were used: citric acid (6, 14, 24), malic acid (25), pyruvic acid (7), α-ketoglutaric acid (4, 26), succinic acid (27), oxalacetic acid (28).

Manometry—The usual manometric procedures were employed except that the substrate was mixed with the enzyme at the start of the experiment to avoid the inactivation which results from shaking the enzyme at 38° in absence of substrate. The manometers were equilibrated for 5 minutes in the bath before the taps were closed.

SUMMARY

The cyclophorase system of rabbit kidney has been shown to catalyze the oxidation of pyruvic acid to carbon dioxide and water by way of the citric acid cycle.

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THE ENZYMATIC STEPS IN UREA SYNTHESIS*

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Investigations on the synthesis of arginine from citrulline (Step II of the Krebs-Henseleit urea cycle) and citrulline from ornithine (Step I) by rat liver homogenates have been reported previously (1–3). The study of the latter reaction was made possible by the apparently critical magnesium ion concentration required for the function of the liver transimination (citrulline to arginine) system. An attempt to isolate these reactions further has led to the use of differential centrifugation methods. By this means a separation of the two reactions has now been effected.

The system for the synthesis of citrulline was found to be associated with the insoluble residues of the rat liver cell, while the system involved in the synthesis of arginine required two fractions, a soluble protein component plus the insoluble residue.

The use of these tissue fractions has led to more detailed information regarding the optimum requirements for Steps I and II. It has now been shown that the citrulline to arginine reaction involves the transfer of hydrogen through the cytochrome system. Furthermore, the step ornithine to citrulline is shown to require the presence of magnesium ions and high concentrations of either ATP (adenosine triphosphate) or AMP (adenylic acid), as well as ornithine, glutamic acid, ammonium, potassium, phosphate, and bicarbonate ions, carbon dioxide, and oxygen at the concentrations used previously with whole homogenate.

It is the purpose of the present paper to describe in detail the experiments leading to the above findings.

Procedures

Preparation of Homogenates—A 20 per cent homogenate in isotonic KCl, prepared as described previously (1), is centrifuged 10 minutes at 2000*g* in a cold room kept at 1°. The resulting cloudy supernatant fluid, used as such or in diluted form, is designated in this study as the *supernatant*. The sediment is washed in a volume of isotonic KCl equal to that of the supernatant, poured off, and centrifuged for another 10 minute period at the same speed. The washed sediment is taken up in the same volume of isotonic KCl and used as such. This preparation is referred to as the

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residue in this paper. 0.5 ml. of residue suspension contained from 1.0 to 1.3 mg. of tissue N.

Homogenates of heart muscle and pigeon breast muscle were prepared in the following manner. The fresh tissue was chilled, cut into small pieces, homogenized in a volume of isotonic NaCl sufficient to make a 25 to 30 per cent homogenate in the Potter and Elvehjem apparatus (4), and filtered through four layers of gauze before use. A volume of 0.5 ml. was used per flask.

Other Tissue Preparations—For freezing treatment, preparations prepared as described above were stoppered tightly and left in a deep freeze compartment overnight. Prior to using they were thawed out at room temperature.

Boiled homogenate preparations were made by heating the residue or supernatant in a steam bath for 10 minutes and filtering off the coagulated proteins. The light greenish yellow filtrate was then used. 0.5 ml. contained from 0.3 to 0.4 mg. of N.

Preparations stored overnight were kept in stoppered tubes at 1°.

An acetone powder of the supernatant was prepared by adding 2 volumes of ice-cold acetone to the supernatant, followed by collection of the precipitate by suction filtration. The residue was dried *in vacuo* over H_2SO_4 and ground in a mortar with isotonic KCl before use.

An acetone powder of whole homogenate was prepared by adding 5 volumes of acetone to the homogenate, followed by centrifugation and desiccation of the residue *in vacuo* over H_2SO_4. The powder was taken up in isotonic KCl before use.

Incubation—All incubations were carried out in Warburg flasks at 38° for 1 hour unless otherwise specified. 1.0 to 1.5 mg. of residue N was used per flask for studies of Step I; 0.5 to 0.8 mg. each of supernatant and residue N for Step II. The total incubation volume was 3.0 ml.

Anaerobic experiments were carried out as described before (3).

Analytical—Methods and procedures for the analyses of urea and citrulline have been described before (1, 3). Urea was measured as the end-product of the citrulline to arginine reaction, since the activity of liver arginase in our preparations was always such as to insure the complete hydrolysis of arginine.

Preparations Used—Sources of the preparations used have been listed previously (1, 3).

Results

Synthesis of Arginine from Citrulline (Step II)

Differential Centrifugation—The results obtained with the use of homogenate fractions prepared by centrifugation are shown in Table I. Neither the residue nor supernatant was active alone, even in the presence of added calf liver arginase (5). Recombination of the two fractions, however, resulted in the regeneration of almost the total activity of the original whole homogenate. Heating of either fraction resulted in the loss of activity. Freezing destroyed only the residue activity. Dialysis against isotonic NaCl was without influence. Acetone powders of the supernatant and whole homogenate showed slight activity when fortified with fresh residue. Both fractions were relatively stable to storage, in contrast to the instability of whole homogenate, which lost two-thirds of its activity on storage for 24 hours at 1°.

Since the function of the insoluble residue could be demonstrated in other tissues such as heart and pigeon breast muscle homogenates, incapable of synthesizing arginine *per se*, it was believed that the residue contained the system for the transport of hydrogen. In addition, the necessity for cytochrome *c*, present in bound form in the residue, has been shown. Since the supernatant from liver is not replaceable by that from other tissues, with the exception of kidney, it is believed to contain the enzyme catalyzing the synthesis. Evidence that this enzyme is present in solution has been obtained in experiments in which a supernatant, prepared by centrifuging a rabbit liver homogenate at 40,000*g* for 2 hours, was found to be active.

Fumarate was included in the incubation medium, since it was found to stimulate the synthesis some 25 per cent. Oxalacetate was equally effective. The function of these oxidizable substrates may be associated with the regeneration of ATP.

While Step I was shown to be sensitive to potassium ions (3), this is not the case with Step II in which sodium or potassium ions were equally effective.

Cytochrome c—The rôle of cytochrome *c* in the transimination reaction was first suggested by Borsook and Dubnoff (6). Our early studies with whole liver homogenate showed its effect to be inconstant (1). The present resolution of homogenate into two parts has now made a study of this question possible. Potter (7) observed that the dissipation of cytochrome *c* took place to a greater extent when water instead of saline was used to homogenize liver. An experiment was devised whereby the sediment from a centrifugation was taken up in water, stored for 10 minutes, and restored to isotonicity. This residue plus the supernatant was pipetted into flasks containing increments of cytochrome *c*. Fig. 1 illustrates the results of this experiment. The optimum cytochrome *c* concentration was about 3×10^{-5} M. A control carried out at the same time with normally prepared residue, with no added cytochrome *c*, gave a value of 70.3 microliters. This would indicate that the normal concentration of cytochrome *c* bound in the residue is of the order of 1.6×10^{-5} M. Supplementation of the supernatant with cytochrome *c* showed no synthesis of arginine from citrulline.

Synthesis of Citrulline from Ornithine (Step I)

Differential Centrifugation—The activity of the system catalyzing this reaction remains in the sediment on centrifugation. Table I shows the relative activities of whole homogenate, regenerated homogenate, and the residue. Although the total yield of citrulline obtained with the residue was low, it was 24 per cent more active than whole homogenate on the basis of mg. of N. The addition of boiled supernatant increased the over-all yield, but not the yield per mg. of N. Additional supplementation of the medium with magnesium ions and adenine nucleotides increased the rate of synthesis as much as 460 per cent at 0.003 M AMP-ATP.

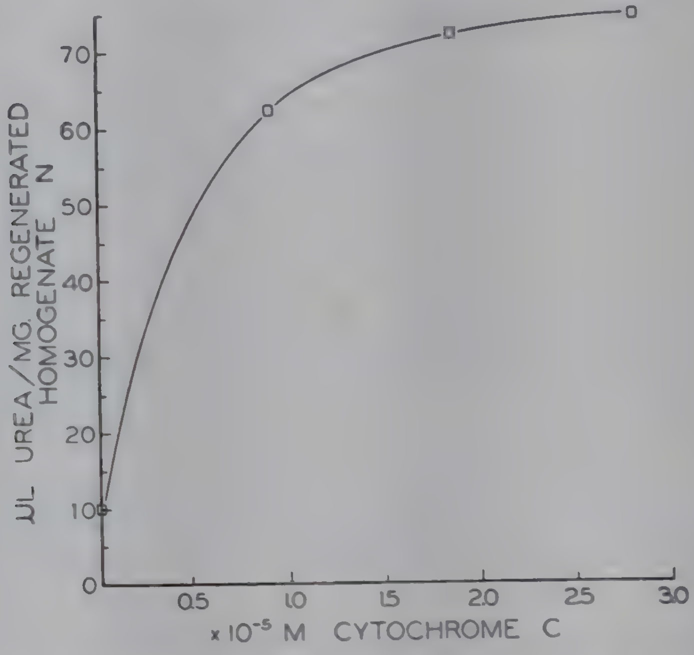

FIG. 1. Effect of cytochrome *c* on the formation of arginine by water-treated homogenate residue. Final substrate concentrations, DL-citrulline 0.0033 M, L-glutamic acid 0.0066 M, ATP 0.001 M, fumarate 0.0066 M, phosphate at pH 7.5, 0.017 M, magnesium ions 0.0033 M, and potassium ions to bring the medium to isotonicity. Gas phase, O_2. 20 per cent KOH in the center wells. Since the preparation contained arginase, arginine was measured as urea.

The stability of the enzyme system to repeated washings was investigated (Table II). The highest activity per mg. of residue N was attained with the first washing. Residue washed two and three times was still somewhat more active than the non-washed preparation. Further washings resulted in considerable loss of activity; thus after six washings, the

TABLE I

Activity of Homogenate Fractions in Steps I and II

Final substrate concentrations for Step I, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.0005 M, ATP 0.0005 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. The supplement for whole homogenate contained no magnesium ions; the supplement for the residue contained 0.01 M magnesium ions. Gas phase, 5 per cent CO_2-95 per cent O_2. Step II, DL-citrulline 0.0033 M, L-glutamic acid 0.0066 M, ATP 0.001 M, fumarate 0.0066 M, phosphate at pH 7.5, 0.017 M, magnesium ions 0.0033 M, and potassium ions to bring the medium to isotonicity. Gas phase, O_2. 20 per cent KOH in the center wells.

	Per cent activity per mg. N	
	Step I	Step II
Whole homogenate (0 time)	100.0	100.0
" " (30 min. storage)	74.0	96.0
Residue + supernatant (30 min. manipulation time)	72.3	94.5
" + supplement optimum for whole homogenate	123.8	2.4
" + boiled supernatant	157.2	4.7
" + supplement optimum for residue	201.1	
Supernatant + supplement optimum for whole homogenate	1.0	1.2
" + heart muscle homogenate		65.5
" + pigeon breast muscle homogenate		45.0

TABLE II

Effect of Repeated Washings on Residue

Final substrate concentrations, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions, 0.0067 M, AMP 0.0005 M, ATP 0.0005 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, magnesium ions 0.01 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO_2-95 per cent O_2. Each washing resulted in the loss of about 10 per cent residue N.

No. of residue washings	Per cent activity remaining per mg. residue N
0	100.0
1	116.4
2	107.4
3	105.0
4	60.2
5	56.6
6	17.5

activity was 17.5 per cent that of the original. The addition of vacuum-concentrated washings and an extract of boiled fresh homogenate (8) did not reactivate the residue. Fortification with cytochrome *c*, biotin, glutathione, coenzyme I, coenzyme II, pyridoxal phosphate, pyridoxine, pyridoxamine, and thiamine pyrophosphate was also without effect.

Requirement of Magnesium Ion—In previous work with whole homogenate (3), magnesium ions were eliminated purposely to prevent the conversion into arginine and urea of the citrulline synthesized. With a lack of magnesium ions the optimum yield obtained was 40 to 50 microliters of citrulline per mg. of whole homogenate N. In the newly devised system the addition of magnesium ions increased the yield 400 per cent, the optimum concentration being 9×10^{-3} M (Fig. 2). It is uncertain whether this effect is due to the function of the ATP regenerating system, or directly associated with the synthesis itself. Manganese ions were without effect.

Requirement of ATP and AMP—Fig. 3 illustrates the requirements of the whole homogenate and residue systems for ATP and AMP. With whole homogenate AMP is at least twice as efficient as ATP. With residue ATP and AMP are almost equally good, and considerably more efficient per unit concentration. The yield of citrulline at the 6 micromole level (0.02 M) was at least 3 times that shown with the whole homogenate system. These effects might be explained on the basis of the requirements of competitive reactions in the case of whole homogenate no longer encountered in the washed residue preparations, or on the basis of the presence of an optimum magnesium ion concentration. Numerous experiments devised to determine the specificity of the system for either ATP or AMP were unsuccessful. While high yields of citrulline could be obtained with high concentrations of these reagents, the final molarity of 0.001 M (AMP 0.0005 M + ATP 0.0005 M) used throughout the study was chosen for reasons of economy.

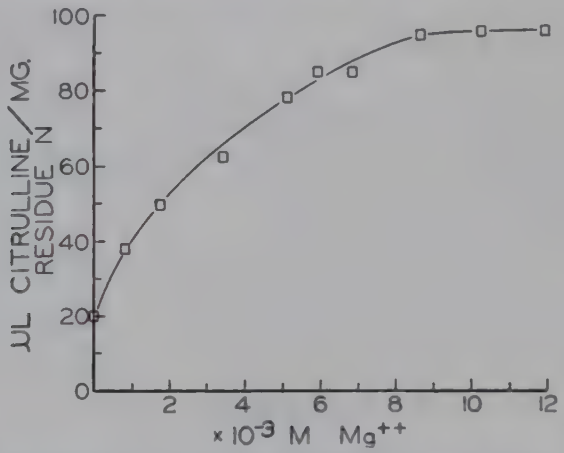

FIG. 2. Effect of magnesium ion concentration on the formation of citrulline by homogenate residue. Final substrate concentrations, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.0005 M, ATP 0.0005 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO_2-95 per cent O_2.

Effect of Incubation Time—In a previous study (3) an unexplained cessation of synthesis was noted after 45 minutes of incubation (Fig. 4). Investigation of this effect with residue showed no stoppage at this time, but instead a linear reaction extending beyond 120 minutes in a system containing 3 micromoles of AMP-ATP. With 9 micromoles of AMP-ATP present, synthesis proceeded very rapidly, the yield being more than twice that obtained with 3 micromoles.

Effect of Tissue Nitrogen—An evaluation of the effect of residue concentration on the synthesis was made (Fig. 5). With increasing concentrations of tissue the theoretical amount of citrulline was almost realized, in contrast to the results obtained with whole homogenate in which higher tissue concentrations stimulated the removal of citrulline. In the presence of 9 micromoles of AMP-ATP, the initial rate of synthesis was somewhat higher than in the case of 3 micromoles. Synthesis with the higher quantity, however, abruptly stopped short of theoretical at higher tissue concentrations. The reason for this is not apparent. It is possible that the higher concentrations of ATP are hydrolyzed with such rapidity as to lower the pH of this system.

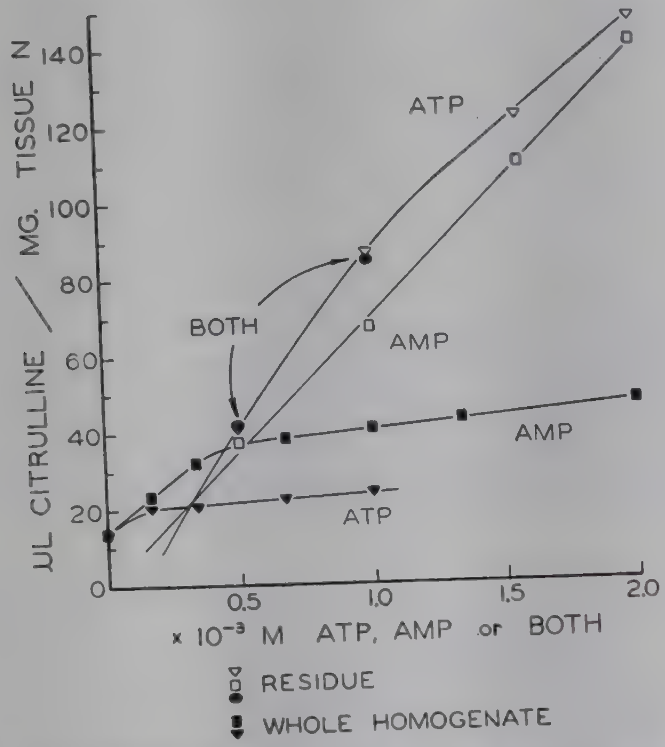

FIG. 3. Effect of increasing concentrations of AMP, ATP, or both on the formation of citrulline by whole homogenate and residue. Final substrate concentrations, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. For experiments with residue, 0.01 M magnesium ions were added. Gas phase, 5 per cent CO_2-95 per cent O_2.

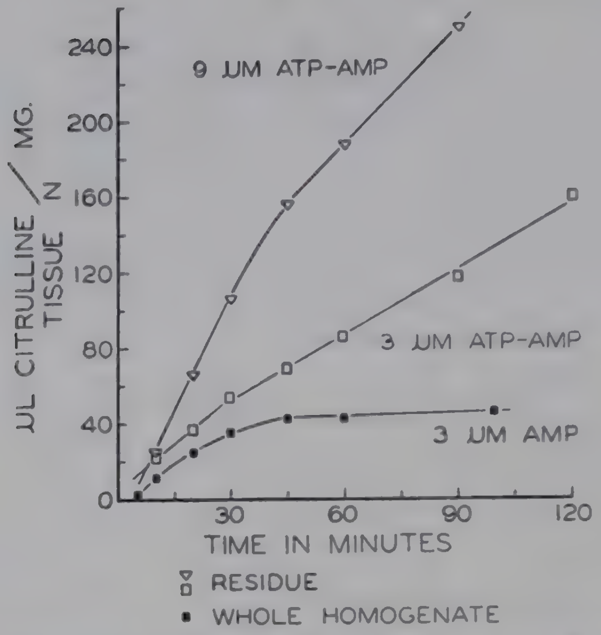

FIG. 4. Effect of incubation time on the formation of citrulline by whole homogenate and residue. Final substrate concentrations for whole homogenate, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.001 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Final substrate concentration for residue same as above except for AMP 0.0005 to 0.0015 M, ATP 0.0005 to 0.0015 M, and magnesium ions 0.01 M. Gas phase, 5 per cent CO_2-95 per cent O_2.

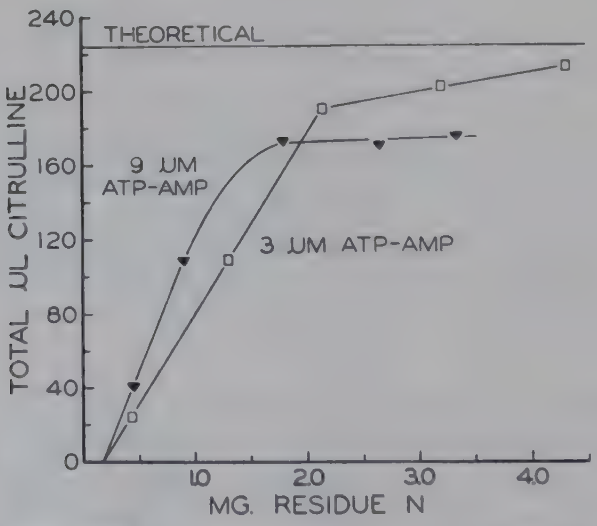

FIG. 5. Effect of tissue concentration on the formation of citrulline by homogenate residue. Final substrate concentrations, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.0005 to 0.0015 M, ATP 0.0005 to 0.0015 M, magnesium ions 0.01 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO_2-95 per cent O_2.

Requirement of Oxygen and Carbon Dioxide—AMP and ATP at high concentrations were incapable of supporting the synthesis of citrulline in an anaerobic atmosphere.

Bach (9) found that formic acid could replace bicarbonate as the source of the urea carbonyl in work with tissue slices. This observation has now been confirmed with homogenates. It also appears that higher concentrations of formic acid can replace both bicarbonate and carbon dioxide.

DISCUSSION

It is now evident that the three steps of the Krebs-Henseleit urea cycle are not as closely related structurally in the whole cell as might be supposed. Step I, the endergonic conversion of ornithine to citrulline, and Step III, the hydrolysis of arginine to urea and ornithine, appear to be functions associated with the nuclear matter of the liver cell (10). Step II, the conversion of citrulline to arginine, also endergonic, appears to be a two-step reaction depending on a soluble cellular component, the specific synthesizing enzyme, as well as the insoluble nuclear residue containing a hydrogen transport system. The latter separation enabled the demonstration of the rôle of cytochrome *c* in the synthesis of arginine. While such a requirement has not as yet been shown for Step I, the possibility cannot be excluded in view of the inhibition of synthesis at low concentrations of cyanide.

A study of the over-all urea cycle with whole homogenate resulted in values never equaling those obtained with liver slices. A few of the factors known to account for the lower magnitude of homogenate results were (1) pH (pH 7.3, intermediate between the optimum pH of the two endergonic reactions (Fig. 6), was used in these studies); (2) the inhibition of the synthesis of arginine by ammonium ions (2) required for the synthesis of citrulline; and (3) the presence of 10 to 20 times the optimum concentration of glutamic acid required for Step II.

The greater effectiveness of AMP over ATP in previous work on the conversion of ornithine to citrulline (3) had led us to believe that the energy-coupling mechanism for that synthesis did not involve ATP. This point is no longer certain. The present work has shown that the synthesis can be accomplished almost equally efficiently with either AMP or ATP. The simple assumption that ATP is active by virtue of its hydrolysis to AMP is shadowed by the apparent requirement of the system for inorganic phosphate and the stimulation of activity by magnesium and fluoride ions. On the other hand, it is equally difficult to assume that the resynthesis of ATP occurs at this rapid pace. The third obvious possibility is that neither is involved as such. Clarification of this question must await further study.

It is highly probable that the conversion of ornithine to citrulline is at least a two-step reaction (11). The centrifugation procedure utilized here did not, however, offer any evidence for the existence of more than one enzymatic system. This may or may not mean that the synthetic steps must of necessity occur simultaneously. It is, however, evident that the differentiation of these steps is requisite for the solution of the AMP-ATP and glutamic acid pictures.

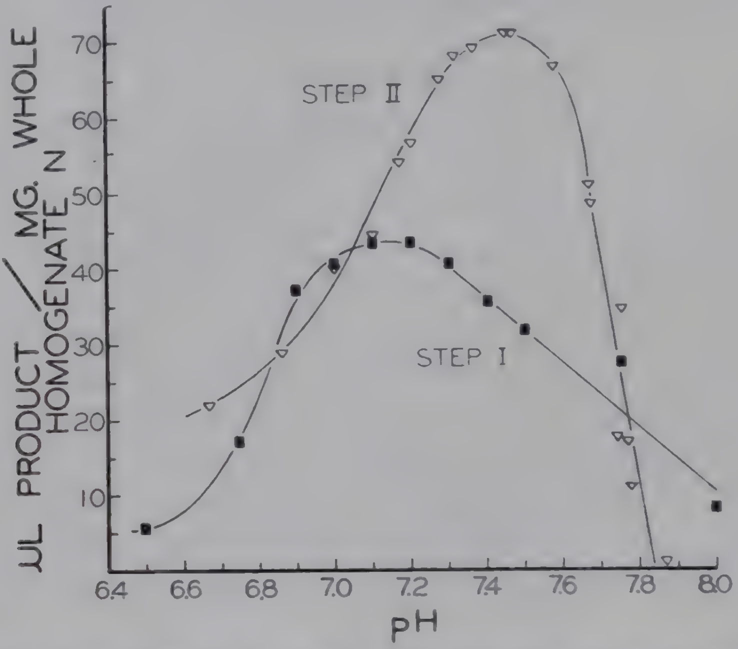

FIG. 6. Effect of pH on Steps I and II of the urea cycle. Final substrate concentrations: *Step I*, DL-ornithine hydrochloride 0.0033 M, L-glutamic acid 0.033 M, ammonium ions 0.0067 M, AMP 0.001 M, phosphate at pH 7.15, 0.017 M, bicarbonate ions 0.0077 M, and potassium ions to bring the medium to isotonicity. Gas phase, 5 per cent CO_2-95 per cent O_2. Whole homogenate N per flask, 2.8 mg. *Step II*, DL-citrulline 0.0033 M, L-glutamic acid 0.0066 M, ATP 0.001 M, fumarate 0.0066 M, phosphate at pH 7.5, 0.017 M, magnesium ions 0.0033 M, and potassium ions to bring the medium to isotonicity. Gas phase, O_2. 20 per cent KOH in the center wells. Tissue N per flask, 1.47 mg. Since the preparation contained arginase, arginine was measured as urea.

An alternative hypothesis involving a "glutamine cycle" for the synthesis of urea has been proposed by Leuthardt and Glasson (12–14). Since no data other than those obtained with the use of liver slices were available, a few experiments with tissue homogenates were carried out. All attempts to demonstrate the synthesis of urea from glutamine were unsuccessful.

SUMMARY

1. Further studies in the synthesis of arginine from citrulline, and citrulline from ornithine, have been carried out with tissue homogenate fractions obtained by differential centrifugation.

2. The enzyme system catalyzing the citrulline-arginine reaction has been resolved into two parts, a soluble synthesizing enzyme fraction and an insoluble hydrogen transport fraction which appears to include the cytochrome system.

3. The enzyme system catalyzing the ornithine-citrulline reaction is associated with the insoluble residue of the liver cell. Supplementation of the residue with magnesium ions in addition to the requirements prescribed for the whole homogenate system is necessary for optimum activity. Both AMP and ATP are almost equally effective with residue.

4. The significance of these findings in relation to the Krebs-Henseleit urea cycle is discussed.

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THE IN VITRO EFFECT OF TOCOPHEROL PHOSPHATE ON THE RESPIRATION OF MUSCLE FROM NORMAL AND DYSTROPHIC RABBITS*

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The observation of Houchin (1) that α-tocopherol phosphate restores to normal the excessive *in vitro* oxygen consumption of muscle of animals made dystrophic by lack of α-tocopherol was the first experimental evidence that this vitamin plays a direct rôle in cellular respiration. Because of the peculiar susceptibility of muscle cells to shredding in the preparation of tissue slices, the procedure that he employed is open to criticism. Confirmation of this claim was therefore attempted by the use of muscle slices and the less objectionable muscle strips from normal and dystrophic rabbits. In our hands, the oxygen uptake of dystrophic muscle strips was greatly in excess of that of normal strips, whereas with muscle slices there was only a slight difference; also in neither case could an *in vitro* influence of tocopherol phosphate on cellular respiration be established.

EXPERIMENTAL

Rabbits weighing 500 to 1000 gm. were placed on the dystrophy-producing diet used in previous studies (1). Symptoms of muscular dystrophy became apparent within 2 to 3 weeks. The animals were used for study when they were unable to right themselves after being placed on their sides. Control rabbits were maintained on the same diet, and were given, orally, 15 mg. of α-tocopherol acetate[1] in olive oil every 4 days.

The rabbits were anesthetized with pentobarbital sodium (35 mg. per kilo of body weight) given by marginal ear vein. The semitendinosus and medial portions of the rectus femoris were rapidly excised and placed in cold Krebs-Ringer-phosphate solution.[2]

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[1] Kindly supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey.

[2] Prepared by mixing in the cold 0.154 M sodium chloride 100 parts, 0.154 M potassium chloride 4, 0.11 M calcium chloride 3, 0.154 M magnesium sulfate 1, 0.1 M sodium phosphate buffer (pH 7.4) 1, and 10 per cent glucose 10, and aerating with oxygen gas for 10 minutes.

With corneal scissors, muscle strips approximately 1 mm. in diameter and 3 to 4 cm. in length were prepared from the chilled semitendinosus by the procedure of Richardson, Shorr, and Loebel (2). Muscle slices were prepared from the rectus femoris in the same manner as that followed by Houchin. Microscopic examination showed that both muscles are equally susceptible to the degenerative processes. The strips and slices thus prepared were kept in chilled Krebs-Ringer-phosphate solution until use. The time elapsing between the biopsy and the beginning of the equilibration period never exceeded 45 minutes.

Selected strips (four or five) and slices (one or two) weighing 200 to 250 mg. were blotted with filter paper, quickly weighed on a torsion balance, and placed in ice-chilled Warburg vessels containing Krebs-Ringer-phosphate solution. In certain experiments 1 mg. of disodium *dl*-α-tocopherol phosphate[1] was added. With alkali in the center well, the flasks were connected to the manometers and equilibrated to 37° for 15 minutes while being flushed with oxygen. Manometric readings were then taken at 15 minute intervals for an hour. After each experiment, the tissue samples were rinsed with distilled water and dried to constant weight at 110°. Oxygen uptake is expressed as Q_{O_2}; *i.e.*, microliters of oxygen consumed per hour per mg. of tissue (dry weight).

RESULTS AND DISCUSSION

The respiration of muscle strips from dystrophic rabbits was roughly double that of normal strips, as indicated in Table I. The oxygen consumption was maintained at a relatively constant rate over the period of an hour. The large standard deviation of the dystrophic muscle strips was expected, since the severity of the dystrophy varied from animal to animal.

The addition of α-tocopherol phosphate did not significantly depress the oxygen consumption of either normal or dystrophic strips. α-Tocopherol phosphate was readily precipitated by the ionic calcium in the suspending medium. Therefore in several experiments calcium salts were omitted from the solution. Even in these cases, α-tocopherol phosphate exerted no effect on the oxygen uptake of normal or dystrophic muscle. While this procedure is obviously open to criticism because of the vital rôle of calcium ions in maintaining the functional integrity of the cell walls, the oxygen uptake was maintained at a constant rate over the time of measurement, indicating that cell destruction was not great.

Similar experiments were conducted with muscle slices from normal and dystrophic rabbits. The oxygen consumption of muscle slices was uniformly lower than that of the corresponding muscle strips; this was especially apparent in the case of dystrophic muscle. The explanation for this lowered respiration probably lies in the greater destruction attending the preparation of muscle slices, and in the difficulty in obtaining slices of uniform dimensions.

Again, as in the case of muscle strips, the *in vitro* addition of tocopherol phosphate to slices had no significant influence on the oxygen consumption of normal and dystrophic slices. The differences fall well within the standard deviation in each case.

These data, therefore, do not confirm the observation that tocopherol phosphate *in vitro* restores to normal the oxygen consumption of dystrophic muscle preparations. Our figures for the oxygen consumption of dystrophic muscle slices are much lower than Houchin's. Perhaps the slice technique in our hands produced greater cell damage. The failure to observe any depression of oxygen uptake of muscle strips may be the result of the inability of tocopherol phosphate to penetrate into the intact muscle cells.

TABLE I

Average Oxygen Consumption of Muscle Tissue from Normal and Dystrophic Rabbits and Effect of Tocopherol Phosphate

Condition	No. of animals	Tocopherol phosphate	Q_{O_2}			
			Interval of reading, min.			
			15	30	45	60
Normal strips	11	−	1.61	1.50	1.51	1.48
			(0.63)*	(0.63)	(0.62)	(0.57)
		+	1.69	1.62	1.59	1.54
			(0.78)	(0.70)	(0.69)	(0.65)
Dystrophic strips	12	−	3.35	3.49	3.39	3.35
			(1.45)	(1.06)	(1.23)	(1.23)
		+	3.21	3.36	3.27	3.21
			(1.39)	(1.30)	(1.17)	(1.25)
Normal slices	8	−	1.33	1.32	1.19	1.20
			(0.53)	(0.51)	(0.47)	(0.45)
		+	0.97	1.04	0.97	0.95
			(0.56)	(0.43)	(0.38)	(0.38)
Dystrophic slices	10	−	1.64	1.55	1.49	1.45
			(0.68)	(0.65)	(0.71)	(0.71)
		+	1.53	1.64	1.54	1.47
			(0.78)	(0.81)	(0.80)	(0.74)

* Standard deviation.

The nature of the rapid and specific action of tocopherol *in vivo* in abolishing the creatinuria and excessive *in vitro* oxygen consumption of dystrophic animals (3, 4) remains obscure. Tocopherol phosphate does not appear to be directly concerned with the succinoxidase system (5). Until the altered metabolism of dystrophic muscle is more clearly understood, further speculation about the physiological rôle of this vitamin is fruitless. With this in mind, the investigation reported in the following paper was undertaken.

SUMMARY

The oxygen consumption of skeletal muscle strips from vitamin E-deficient dystrophic rabbits was approximately double that of muscle strips from normal rabbits. The oxygen consumption of muscle slices was much lower than that of the corresponding muscle strips, especially in the case of dystrophic muscle.

In contrast to previous reports from this laboratory, the addition of α-tocopherol phosphate *in vitro* had no significant effect on the respiration of muscle strips or slices, either from normal or dystrophic animals.

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OXIDATIVE PHOSPHORYLATION PROCESSES IN NUTRITIONAL MUSCULAR DYSTROPHY*

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Nutritional muscular dystrophy is characterized by a pronounced loss of contractile power which appears to precede the degeneration of the muscle fibers (1). This paralysis may be due either to an impairment of the contractile mechanism as evidenced by the disappearance of the cross striations or to an impairment in energy utilization. Since the energy for muscle contraction is thought to be mobilized by oxidative phosphorylation mechanisms, this study was undertaken to discover whether any such processes may be affected in dystrophy, specifically the phosphorylation of creatine associated with the oxidation of carbohydrate.

If the oxidation of α-glycerophosphate and fructose-1,6-diphosphate is assumed to proceed entirely through the so called Meyerhof-Embden-Parnas pathway (2) to pyruvic acid, the transfer of hydrogen from the substrate at each oxidative step may proceed either aerobically through the coenzyme I-flavin-cytochrome systems to molecular oxygen or anaerobically by means of the coenzyme I-linked dismutation with pyruvic acid to form lactic acid. Two energy-rich phosphate bonds, transferable to creatine through the adenylic system, would be formed per mole of triose phosphate oxidized to pyruvic acid, but only one would be formed under fluoride inhibition. Phosphorylation was therefore studied by measurement of the phosphocreatine formed from creatine, and oxidation by the oxygen consumed and lactic acid formed.

EXPERIMENTAL

Muscular dystrophy was produced in hamsters by the same procedure as previously described (3). It was also produced in guinea pigs placed on the same diet, with the oral administration of 10 mg. of ascorbic acid every other day. In this species, dystrophy became acute after about 5 weeks and was characterized by a very severe paralysis, loss in weight, and sudden death. Control animals, maintained on the same diet, were given ascorbic acid as above and 15 mg. of tocopherol acetate[1] every 4 days. No symp-

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* Aided by a grant from the John and Mary R. Markle Foundation.

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[1] Kindly supplied by Hoffmann-La Roche, Inc.

toms of the guinea pig "wrist-stiffness" described by van Wagtendonk and Wulzen (4) were observed.

The method of preparing distilled water homogenates of thigh muscle has been described (3). Each Warburg flask contained, besides the indicated substrate in the side arm, the following in micromoles: creatine 230, potassium chloride 400, magnesium chloride 20, nicotinamide 20, coenzyme I 0.75, adenosine triphosphate 1.33, and sodium phosphate buffer (pH 7.4) 10, and 100 mg. of fresh muscle (as 10 per cent muscle homogenate) in a total volume of 3.0 ml. The center well contained 0.1 ml. of 2 N sodium hydroxide. In the aerobic experiments, each flask also contained 8×10^{-8} moles of cytochrome *c* in an air atmosphere. In the anaerobic experiments, each flask contained 10 micromoles of lithium pyruvate[2] under a nitrogen atmosphere. In certain experiments, 5 micromoles of sodium fluoride were added to inhibit the utilization of 2-phosphoglyceric acid.

Cytochrome *c* was prepared according to the method of Keilin and Hartree (5), as modified by Potter (6). Coenzyme I of about 40 per cent purity was prepared from starch-free bakers' yeast by the method of Williamson and Green (7). The barium salt of adenosine triphosphate (ATP) was prepared by the method of Needham (8), as modified by DuBois, Albaum, and Potter (9). The product was 91 per cent pure, as measured by 7 minute hydrolysis. All additions were adjusted to pH 7.4 and employed at concentrations optimum for maximum phosphorylation.

All incubations were conducted at 37°. Manometric readings were taken 20 minutes after the substrate was tipped in from the side arm. The flask contents were then quickly chilled to 0° and deproteinized with trichloroacetic acid, according to the procedure outlined by Potter (10). The neutralized filtrate was analyzed for phosphocreatine by an adaptation (10) of the method of Fiske and Subbarow (11), and for triose phosphate by 30 minute hydrolysis in 1 N sodium hydroxide at 25°, followed by the usual colorimetric determination for inorganic phosphate. Lactic acid in the filtrate was determined by the procedure of Barker and Summerson (12).

The data reported are net changes obtained by subtracting the values for control flasks which lacked only substrate. They are expressed as micromoles per hour, except those for oxygen consumption, which, in order to be on the same basis as lactic acid data, are expressed as microatoms per hour. In computing phosphocreatine to oxygen ratios (P to oxygen), the corresponding anaerobic phosphocreatine to lactic acid ratio (P to lactic) is assumed to prevail under aerobic conditions. Therefore

$$\text{P:oxygen} = \frac{(\text{P}_{\text{aer.}}) - (\text{lactic}_{\text{aer.}}) \times ((\text{P}_{\text{anaer.}}) : (\text{lactic}_{\text{anaer.}}))}{(\text{Oxygen})}$$

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[2] Generously furnished by Dr. R. M. Featherstone of the Department of Pharmacology.

RESULTS AND DISCUSSION

With normal guinea pig muscle and glycerophosphate as substrate, as shown in Table I, the addition of fluoride reduced the formation of lactic acid and phosphocreatine by about half, although the oxygen consumption was only slightly diminished.

The P to oxygen ratio is $(8.2 - 8.0 \times 0.45)/6.0$ or 0.77 in the absence of fluoride, and by similar calculation it is 0.70 in the presence of fluoride. According to theory, the former value is too small, due probably to the "leak" of energy-rich bonds by ATPase action. Under anaerobic conditions, however, less than half a mole of phosphocreatine was formed per mole of lactic acid, without fluoride, and even less in its presence. The further oxidation of pyruvic acid may account in part for the lack of agreement between the aerobic and anaerobic phosphorylation ratios. In the presence of fluoride, however, this disparity demands a different explanation; in such cases, the phosphorylation efficiency may possibly depend upon the potential gradient through which the hydrogen transport system is operative (13).

TABLE I

*Phosphorylation of Creatine from Oxidation of Glycerophosphate by Guinea Pig Muscle Homogenate**

Oxygen uptake is expressed as microatoms per hour. Lactic acid, phosphocreatine, and triose phosphate are expressed as micromoles per hour. The ratios in the last two columns are explained in the text.

Condition	Gas	Fluoride	Oxygen uptake	Lactic acid formed	Phosphocreatine formed	Triose phosphate formed	P:lactic	P:oxygen
Normal (7 animals)	O_2	−	6.0	8.0	8.2	0.6		0.77
	"	+	5.0	2.8	4.0	0.6		0.70
	N_2	−		22.3	10.0	0.2	0.45	
	"	+		12.3	4.8	0.1	0.39	
Dystrophic (8 animals)	O_2	−	6.1	3.3	1.6	1.3		0.13
	"	+	5.5	0.5	1.0	1.3		0.16
	N_2	−		15.0	3.8	0.8	0.25	
	"	+		13.4	2.4	0.6	0.18	

* Each flask contained the reaction mixtures described in the text, with 100 micromoles of sodium glycerophosphate (Eastman) as substrate.

The differences between normal and dystrophic guinea pig muscle are striking. In dystrophic muscle, the aerobic formation of lactic acid and phosphocreatine was very small, although the oxygen consumption was equal to that of normal muscle. This is reflected in the sharp reduction of the P to oxygen ratios in the case of dystrophic muscle. Under anaerobic conditions, lactic acid was less than in the normal, but the efficiency of oxidative phosphate transfer was markedly diminished, as judged by the much smaller P to lactic ratio. Addition of fluoride lowered this still further. In all cases, the amounts of triose phosphate formed were small, but the slight accumulation with dystrophic muscle may be significant.

Normal hamster muscle produced much less phosphocreatine than normal guinea pig muscle, although the oxygen consumption was very much greater. As seen in Table II, oxygen consumption and lactic acid and phosphocreatine formation were all diminished in dystrophic hamster muscle. Addition of fluoride lowered the phosphocreatine synthesis to almost zero without noticeably affecting either the aerobic or anaerobic oxidations. As with guinea pig muscle, triose phosphate values were small, but there appeared to be a slight accumulation with dystrophic muscle.

TABLE II

*Phosphorylation of Creatine from Oxidation of Glycerophosphate by Hamster Muscle Homogenates**

Condition	Gas	Fluoride	Oxygen uptake	Lactic acid formed	Phosphocreatine formed	Triose phosphate formed	P:lactic	P:oxygen
Normal (6 animals)	O_2	–	14.4	4.3	2.2	1.6		0.08
	"	+	13.8	2.0	1.2	2.0		0.06
	N_2	–		18.4	4.5	0.4	0.24	
	"	+		15.9	2.6	0.5	0.16	
Dystrophic (5 animals)	O_2	–	7.9	3.4	1.4	2.3		0.11
	"	+	7.7	0.9	0.4	2.2		0.04
	N_2	–		6.9	1.0	0.4	0.15	
	"	+		6.4	0.6	0.9	0.09	

* Each flask contained the reaction mixture described in the text, with 100 micromoles of sodium glycerophosphate as substrate. The data are expressed in the same terms as those used in Table I.

The oxidation of fructose-1,6-diphosphate presents a somewhat different picture. With guinea pig muscle, as shown in Table III, fluoride inhibited the formation of lactic acid and phosphocreatine under aerobic and anaerobic conditions with both normal and dystrophic muscle, but stimulated the oxygen uptake, probably by preventing the formation of pyruvate which would act as a hydrogen acceptor. A disparity between the aerobic and anaerobic phosphorylation, similar to that seen with glycerophosphate oxidation, also exists with normal fructose-1,6-diphosphate oxidation.

The differences between normal and dystrophic guinea pig muscle were not as great with fructose-1,6-diphosphate as with glycerophosphate as substrate. Under aerobic conditions, lactic acid and phosphocreatine were somewhat diminished in dystrophy, but oxygen uptake was unaltered. A comparison of the P to oxygen ratios, however, shows that dystrophy significantly reduced the aerobic phosphorylation efficiency. Under anaerobic conditions, the P to lactic ratios were in no way diminished in dystrophy.

TABLE III

*Phosphorylation of Creatine from Oxidation of Fructose-1,6-diphosphate by Guinea Pig Muscle Homogenate**

Condition	Gas	Fluoride	Oxygen uptake	Lactic acid formed	Phospho-creatine formed	Triose phosphate formed	P:lactic	P:oxygen
Normal (6 animals)	O_2	−	2.2	32.5	14.9	14.0		2.18
	"	+	3.2	11.5	9.3	14.4		1.81
	N_2	−		42.6	13.2	12.7	0.31	
	"	+		26.4	7.8	14.5	0.30	
Dystrophic (5 animals)	O_2	−	2.5	24.4	12.2	20.1		0.48
	"	+	3.6	7.6	5.8	20.3		1.00
	N_2	−		34.9	15.6	17.3	0.45	
	"	+		26.2	7.7	20.1	0.29	

* Each flask contained the reaction mixture described in the text, with 30 micromoles of fructose-1,6-diphosphate (Schwartz) as substrate. The data are expressed in the same terms as those used in Table I.

TABLE IV

*Phosphorylation of Creatine from Oxidation of Fructose-1,6-diphosphate by Hamster Muscle Homogenate**

Condition	Gas	Fluoride	Oxygen uptake	Lactic acid formed	Phospho-creatine formed	Triose phosphate formed	P:lactic	P:oxygen
Normal (5 animals)	O_2	−	9,5	44.7	4.8	12.6		0.08
	"	+	12.2	8.0	5.3	16.2		0.04
	N_2	−		71.8	6.8	10.7	0.09	
	"	+		33.3	3.1	14.9	0.09	
Dystrophic (5 animals	O_2	−	2.6	11.7	3.0	22.1		0.35
	"	+	4.0	7.8	1.4	21.6		0.09
	N_2	−		29.2	5.3	19.3	0.18	
	"	+		24.2	3.7	21.0	0.15	

* Each flask contained the reaction mixture described in the text, with 30 micromoles of fructose-1,6-diphosphate as substrate. The data are expressed in the same terms as those used in Table I.

The oxidation of fructose-1,6-diphosphate by hamster muscle, as shown in Table IV, was similar to that by guinea pig muscle, except that oxygen consumption, lactic acid, and phosphocreatine were all diminished in dystrophy, with the result that changes occur in the phosphorylation efficiencies which are difficult to evaluate. Under anaerobic conditions, the formation of lactic acid by dystrophic muscle was very much smaller, although surprisingly the phosphocreatine formation was not greatly different from the normal.

Since, with dystrophic guinea pig muscle, neither the oxygen uptake nor the anaerobic formation of lactic acid was greatly altered from the normal, it follows that the oxidations to the 1,3-diphosphoglyceric acid step are probably not impaired in dystrophy. In the case of glycerophosphate, however, the subsequent phosphorylation steps do appear to be impeded, the evidence being the lowered phosphocreatine formation, and, in the aerobic oxidation without fluoride, the lowered lactic acid formation. This lactic acid could come only from the pyruvic acid produced. If the slightly increased triose phosphate values have significance, this is additional evidence of such a barrier.

Disturbances in phosphorylation may be general in the oxidative metabolism of dystrophic muscle. Lu *et al.* (14) found that the ability of dystrophic muscle to phosphorylate glycogen was reduced by half. Boyer (15) similarly found that dystrophic muscle lost the ability to derive phosphate bond energy from the oxidation of certain members of the tricarboxylic acid cycle. His data indicated that there was no impairment in the transfer of energy-rich phosphate bonds from enol-phosphopyruvic acid to creatine in dystrophy.

The differences between the phosphorylation efficiencies of fructose-1,6-diphosphate and glycerophosphate are not explained if both are oxidized by the same pathway in the guinea pig. The reason for the differences in their oxidation is more apparent; 1 mole of fructose-1,6-diphosphate yields 2 moles of pyruvic acid; 1 mole of glycerophosphate yields only 1 mole of pyruvic acid. Therefore, if primed by a very small amount of pyruvic acid, the anaerobic oxidation of fructose-1,6-diphosphate may theoretically proceed entirely to lactic acid, according to the well known mechanism for anaerobic glycolysis. Since, however, glycerophosphate requires two dehydrogenation steps, at least half of the oxidation must proceed aerobically unless an excess of pyruvic acid is already present. Thus the greater tendency of fructose-1,6-diphosphate oxidation to proceed anaerobically may be explained.

The large accumulation of triose phosphate with fructose-1,6-diphosphate as substrate probably results from the high activity of muscle aldolase (16). The greatly increased triose phosphate values with dystrophy may be due either to a barrier in the system or to an increased aldolase activity. With glycerophosphate, triose phosphate formation is limited by the rate of the initial oxidation step and thus accumulates in small amounts.

Dystrophy in hamsters impeded both oxidation and phosphorylation in the utilization of either substrate; oxygen uptake, lactic acid, and phosphocreatine formation were all diminished.

These alterations would seem to be an indirect or subsidiary effect of the avitaminosis because the *in vitro* addition of α-tocopherol phosphate did not restore normal activity to dystrophic muscle homogenates. Furthermore, in severely dystrophic rabbit muscle, a similar impairment in oxidative phosphorylation could not be demonstrated. It would seem that the action of tocopherol *in vivo* is mediated through as yet unrecognized agencies which are lost *in vitro*, and which may vary from species to species. These species differences make it difficult to generalize about the metabolic disturbances encountered in nutritional muscular dystrophy.

TABLE V

*Adenosinetriphosphatase Activity of Normal and Dystrophic Hamster and Guinea Pig Muscle**

Animal	Ca^{++}	Normal	Dystrophic
Hamster	−	13.2	8.5
	+	21.6	14.7
Guinea pig	−	12.8	7.6
	+	16.1	11.0

* Expressed as γ of P split from ATP per mg. of tissue (wet weight) in 15 minutes at 37°, pH 7.4, corrected for phosphate content of the tissue and for non-enzymatic ATP hydrolysis.

Finally, in a comparison of normal and dystrophic muscle, it must be emphasized that there is a smaller active mass of muscle as the result of necrosis and fibrotic infiltration which may, in part, explain the lowered enzymatic activity in advanced dystrophy. There was a fair parallelism between the lowered oxidative phosphorylation and the muscle degeneration observed histologically in sections of the same muscle.[3]

As possible indices of cellular destruction, assays of ATPase activity (adenylpyrophosphatase (17)) according to the micromethod of DuBois and Potter (18) were carried out in triplicate on the same tissue homogenates as were used in the phosphorylation experiments. Although the method may provide only an approximation, an appreciable destruction of ATPase had taken place in dystrophic muscle, as shown in Table V.

The activation of ATPase by the high calcium content of dystrophic muscle has been suggested by Morgulis and Jacobi (19) as a cause for the high respiration through the destruction of ATP and the accompanying increase in inorganic phosphate. In our hands, 0.003 M calcium chloride, sufficient to give complete activation, stimulated the ATPase of normal and dystrophic muscle to the same extent. The extra calcium of dystrophic muscle is probably un-ionized and incapable of stimulating ATPase. Since ATPase is believed to be intimately associated with myosin (20), dystrophy appears to involve an alteration of the contractile structure of the muscle as well as an impairment in the utilization of energy for contraction.

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[3] Sections were made by the Department of Pathology.

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SUMMARY

The phosphorylation of creatine by normal and dystrophic hamster and guinea pig muscle homogenates was studied in the aerobic and anaerobic oxidation of glycerophosphate and fructose-1,6-diphosphate in the presence of the necessary known cofactors.

With glycerophosphate as substrate, the aerobic and anaerobic oxidation rate by dystrophic guinea pig muscle was little different from the normal, but the coupled phosphorylation of creatine was greatly diminished. With fructose-1,6-diphosphate, the effects of dystrophy were similar but less marked. Dystrophy appeared to affect the aerobic phosphorylation more than the anaerobic.

In dystrophic hamster muscle homogenates, both oxidation and phosphorylation processes were impeded.

These alterations appear to be indirect effects of the vitamin E deficiency, but may partially explain the associated paralysis.

Dystrophy in hamsters and guinea pigs markedly lowers the muscle adenosinetriphosphatase activity and is interpreted to mean that the contractile structure in dystrophic muscle is also impaired.

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MOLYBDENUM IN THE NUTRITION OF THE RAT*

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Some years ago a group of experimentalists (1–3) observed that the scouring of cattle on the so called "teart" pastures of England could be traced to the high molybdenum content of the soil and herbage. Feeding molybdenum salts reproduced the symptoms. Subsequently it was found that feeding copper sulfate as a drench prevented or cured the condition. Apparently a similar high molybdenum area is present in California (4). In Australia, further evidence of the rôle of copper in molybdenosis was discovered (5). Molybdenum fed to sheep and cattle caused loss of liver copper even when the copper intake in the diet was increased.

Recently several other interesting interactions between nutritional elements have appeared. Thus it was found (6) that copper will counteract the anemia caused by feeding zinc to rats. It is also claimed (7) that arsenic and manganese fed in addition to iron and copper salts will allow greater hemoglobin production in rats on a milk diet than when only iron and copper are added as the mineral supplement. It is clear from these studies that the toxicity, deficiency, metabolism, etc., of one element, at least in herbivorous animals, may be profoundly influenced by the levels of certain of the other elements of the diet.

Molybdenum has been found capable of combining with catechol in a 1:2 molar ratio (8). It was shown that such a combination reduced the toxicity of catechol for bacteria, thus allowing proliferation of the microorganisms in an otherwise toxic concentration of catechol. It was suggested that these reactions could account for the diarrhea seen in cattle suffering from molybdenosis.

This paper shows that molybdenum in the form of sodium molybdate retards the growth of rats on a purified ration. The molybdenum toxicity can be overcome by feeding larger amounts of copper as copper sulfate or by the incorporation into the diet of 5 per cent whole liver substance but not by feeding higher levels of iron, zinc, or cobalt. In another experiment the fate of a given dose of molybdenum (labeled with radioactive Mo^{99}) when fed either alone or with copper or catechol was investigated.

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EXPERIMENTAL

In a preliminary experiment a number of 21 day-old, male, white Sprague-Dawley rats were placed on the following purified ration: alcohol-extracted casein 18 per cent, corn oil 5 per cent, sucrose 73 per cent, Salts IV 4 per cent. To 1 kilo were added 10 ml. of a vitamin solution containing per liter 0.6 gm. of thiamine hydrochloride, 0.6 gm. of riboflavin, 4.0 gm. of calcium pantothenate, 0.4 gm. of pyridoxine hydrochloride, 200 gm. of choline chloride, 4.0 gm. of nicotinic acid, 20 gm. of inositol, 50 gm. of *p*-aminobenzoic acid, 50 mg. of folic acid, and 20 mg. of biotin. The fat-soluble vitamins were given twice each week with a dropper. To this diet Mo in the form of $Na_2MoO_4 \cdot 2H_2O$ was added to give final concentrations in the diet of 0.00 (control), 50, 100, and 500 mg. per cent of Mo.

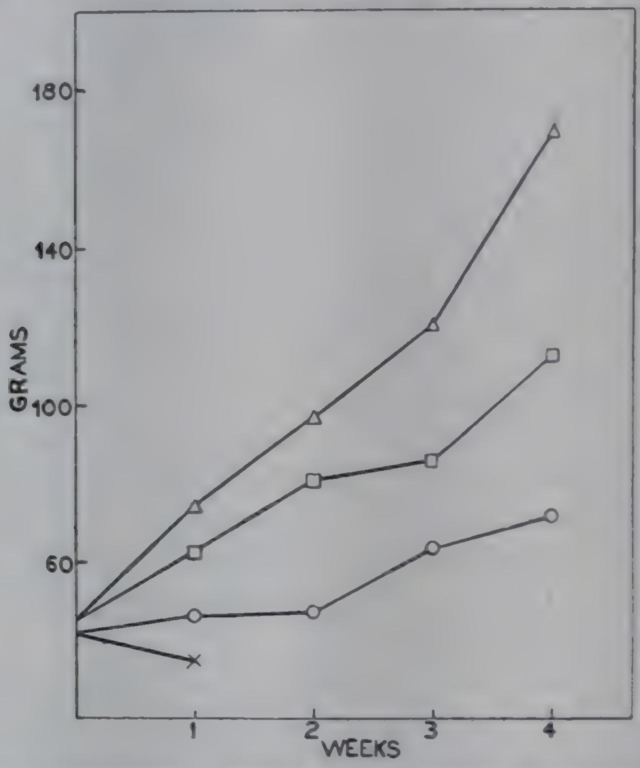

Fig. 1. Growth retardation in rats fed a purified diet containing molybdenum ($Na_2MoO_4 \cdot 2H_2O$). △, diet without Mo (control); □, diet contained 50 mg. per cent of Mo; ○, diet contained 100 mg. per cent of Mo; ×, diet contained 500 mg. per cent of Mo.

Fig. 1 shows the growth curves for these rats over a 4 week period. All four rats receiving 500 mg. per cent of Mo died shortly after the 1st week. There was no indication of diarrhea and an autopsy did not reveal any gross abnormalities in individuals from any group other than extreme emaciation. Further, a blood examination by the routine methods gave an apparently normal picture for red blood cells, white blood cells, hematocrit, and hemoglobin.

From consideration of the growth curves reproduced in Fig. 1, it was decided that for an experiment designed to test the counteraction of molybdenum toxicity a series of rats fed a level of 40 mg. per cent of Mo should prove satisfactory.

In view of the remarkable, and somewhat puzzling, interaction among various nutritional elements which was described briefly above, it was decided to test the effect of copper and iron alone and then, in a single experiment, to include copper, iron, zinc, and cobalt in rations containing 40 and 80 mg. per cent of Mo. The copper was added as $CuSO_4 \cdot 5H_2O$, the iron as $Fe_2(SO_4)_3$, the zinc as $ZnCl_2$, and the cobalt as $CoCl_2 \cdot 6H_2O$. The effect of whole liver substance was also investigated. The basal ration was identical to that used above and was again fed *ad libitum* to four rats in each group. In this ration the various substances were intimately mixed in the concentrations indicated in Fig. 2, which shows the growth response of the animals receiving 40 mg. per cent of Mo.

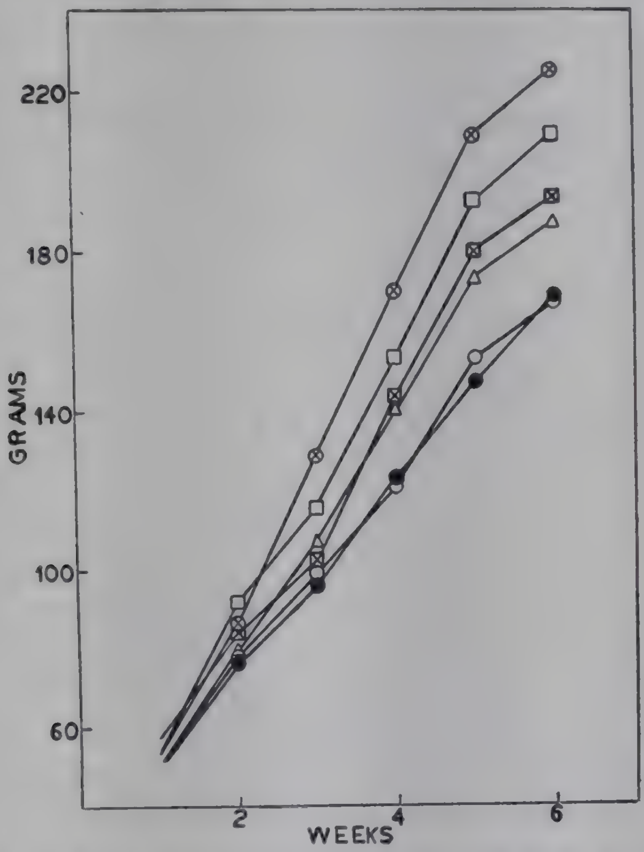

FIG. 2. Growth effect in rats of various minerals and whole liver substance added to a purified diet containing 40 mg. per cent of molybdenum ($Na_2MoO_4 \cdot 2H_2O$). ⊗, 40 mg. per cent of Mo + 5 per cent whole liver substance + basal; □, purified basal (control); ⊠, 40 mg. per cent of Mo + 2 mg. per cent of Cu + basal; △, 40 mg. per cent of Mo + 2 mg. per cent of Cu + 20 mg. per cent of Fe + 20 mg. per cent of Zn + 2 mg. per cent of Co + basal; ○, 40 mg. per cent of Mo + 20 mg. per cent of Fe + basal; ●, 40 mg. per cent of Mo + basal.

It is apparent that 5 per cent whole liver substance contained some factor which was absent from the purified ration. Most recent work on the nutrition of the rat supports this view (see the paper by Jaffé and Elvehjem (9)). The addition of iron alone did not prevent the toxicity of molybdenum. However, the addition of 2 mg. per cent of Cu either alone or together with iron, zinc, and cobalt gave an average gain of 28 gm. at the end of 6 weeks. The weights of the individual rats receiving 40 mg. per cent of Mo plus 2 mg. per cent of Cu plus the basal diet were 185, 189, 194, and 203 gm., while those receiving 40 mg. per cent of Mo plus the basal were 150, 166, 170, and 182 gm. at the end of this period.

The group receiving 5 per cent whole liver substance plus 80 mg. per cent of Mo again showed no growth retardation. The remaining rats fed this level of molybdenum grew very poorly, including those fed 2 mg. per cent of Cu.

It was next decided to attempt to trace, as quantitatively as possible, the fate of a given dose of molybdenum and to study the influence of a simultaneous dose of copper or catechol. Although various methods are available for the determination of molybdenum in biological materials (10), the use of a radioactive tracer has an obvious advantage in that the preexisting molybdenum in the various tissues does not interfere. Further, the detection of a radio element leaves little to be desired from the point of view of sensitivity of measurement. A sample of Baker's molybdic oxide (MoO_3) which had been irradiated with neutrons was obtained from the Monsanto Chemical Company. The sample assayed 99.9 per cent MoO_3 and contained the following impurities: SO_4 0.003 per cent, PO_4 0.0005 per cent, insoluble in NH_4OH 0.007 per cent. In the irradiation process apparently only the stable Mo^{98} isotope undergoes a nuclear reaction (n, γ) to give the radioactive Mo^{99} isotope. This element decays with a 1.3 m.e.v. β and 0.77, 0.82, 0.84 m.e.v. γ emission to technetium $({}_{43}Tc^{99})$. The half life is 67 hours.

The sample of radioactive molybdic acid (10.0000 gm.) was dissolved in a stoichiometric amount of concentrated sodium hydroxide and the neutral solution diluted to 50 ml. From this master solution various subdilutions were prepared for feeding experiments and for constructing the counting standards.

The element was introduced into the back of the throat in 0.5 ml. of solution from a hypodermic syringe fitted with a blunt needle. In the first experiment twelve rats were each given 13.34 mg. of the radiomolybdenum. Six of these were then immediately given 0.5 ml. of a solution of copper sulfate containing 5.00 mg. of copper. After intervals of approximately $2\frac{1}{2}$ hours, 1 day, and 2 days, respectively, two rats from each group were sacrificed, and the tissues weighed in tared porcelain crucibles and ashed in a muffle furnace after carmelizing at 100°. The ash was weighed, dissolved or suspended as evenly as possible in concentrated hydrochloric acid, and an aliquot transferred to a 50 mm. lead dish. The samples were dried at 100° and their radioactivity measured with an Instrument Development Laboratories counter. The activity was corrected for decay and ash absorption. The latter correction was obtained from a curve representing per cent recovery per mg. of ash. The urine and feces, as well as the various tissues, were counted for those rats on the test 2 days. The results are given in Table I.

In a second experiment twice as much molybdenum was fed (26.68 mg.) to three rats. To two of these was then immediately given 0.5 ml. of a solution of catechol containing 6.09 mg. of the alcohol. According to the theory discussed above (8) this should suffice to combine with approximately 10 per cent of the ingested molybdenum. Higher levels of catechol are toxic. The three rats were sacrificed after 1 day and the tissues, feces, and urine counted in the usual way. The results are shown in Table II.

It is evident that a considerable portion of the ingested Mo is rapidly absorbed and rapidly excreted. The high levels in the feces show that much of the element may never leave the alimentary canal. Although the animals were fasted for 24 hours prior to administration of the molybdenum, the varying amounts of food in the stomach and intestine may account for the variable degrees of absorption. Aside from the stomach and intestine, the tissues followed a fairly regular scheme of rather rapid elimination of the element. The figures in Table I do not show any obvious effect of copper fed in this way. In Table II there is some indication that the feeding of catechol may delay the elimination of the element. After 2 days (Table I) the highest concentration of molybdenum was in the bones and kidneys. This is in agreement with certain experiments on inert molybdenum (11).

Since, as the above experiment illustrates, a considerable fraction of the molybdenum is not absorbed, there is a very real possibility that it has formed a less soluble complex with certain substances in the alimentary canal. On the other hand molybdenum could probably combine with other substances in such a way that its toxicity would be lowered without appreciably influencing its rate of absorption and elimination. Copper and molybdenum may form the rather poorly characterized insoluble copper molybdate. In a study of the precipitation of molybdate (12) it was found that in the potentiometric titration of copper sulfate with sodium molybdate a precipitate began to form at pH 5.28. The composition of the copper molybdate precipitate was found to be 1.0 CuO:0.7 MoO_3; *i.e.*, Cu 35.25 per cent, Mo 37.24 per cent.

TABLE I*

Distribution of Radioactive Molybdenum in Tissues of Rats Fed Molybdenum Alone and with Copper

Group No.	1	2	3	4	5	6
Average weight, *gm.*	132.6	150.3	142.9	155.8	96.2	71.4
Time on test, *hrs.*	2.1	26.1	51.0	2.5	26.4	51.4
Mo dose, *mg.*	13.34	13.34	13.34	13.34	13.34	13.34
Cu " "				5.00	5.00	5.00
Total per tissue						
	γ	γ	γ	γ	γ	γ
Stomach	1536.89	46.40	6.78 (0.12)	45.86	133.25	18.23 (0.29)
Kidneys	28.46	11.85	7.12 (0.13)	22.60	9.77	4.45 (0.07)
Liver	60.17	8.78	7.01 (0.13)	45.43	8.65	6.75 (0.11)
Intestine	214.97	365.17	72.73 (1.31)	161.05	584.75	91.35 (1.46)
Bone	4.07	1.63	0.98 (0.02)	4.14	2.25	1.31 (0.02)
Heart	4.16	0.83	0.61 (0.01)	2.53	0.85	0.58 (0.01)
Lungs	12.40	1.27	1.12 (0.02)	13.29	2.21	1.23 (0.02)
Carcass	811.00	377.50	80.57 (1.45)	950.60	270.35	84.14 (1.35)
Feces			697.50 (12.53)			1364.70 (21.75)
Urine			4692.00 (84.30)			4701.00 (74.92)
Recovery, %	20.03	6.11	41.73	9.34	7.59	47.03
Per gm. tissue						
Blood	28.83	2.34	1.81	13.16	3.04	2.34
Stomach	176.36	5.80	2.94	9.26	19.26	4.64
Kidneys	18.47	7.68	7.14	14.34	9.74	2.80
Liver	9.07	1.23	0.84	5.44	1.71	1.65
Intestine	13.88	25.42	6.19	9.02	25.52	14.43
Bone	10.89	4.79	3.03	10.27	6.38	3.88
Heart	9.00	1.50	1.02	10.00	1.94	1.63
Lungs	15.81	1.57	1.16	12.40	3.39	2.16
Carcass	8.53	3.25	0.63	8.03	3.87	1.49
Feces			290.91			597.44

* Each column is the average from two rats. The figures in parentheses are the per cent of total Mo recovered.

Approximately 50 gm. of pure white anhydrous copper sulfate was dried at room temperature for 3 days over calcium chloride. Exactly

44.21 gm. of this salt were weighed and diluted to 100 ml. with distilled water. 10 ml. of this solution contained 1.7613 gm. of copper. Exactly 10 gm. of molybdic oxide containing Mo^{99} were dissolved in sodium hydroxide and the stoichiometric amount of sodium hydroxide added. The neutral solution, after dilution to 50 ml., contained 1.334 gm. of molybdenum per 10 ml.

10 ml. of the copper solution were added dropwise with occasional stirring to 10 ml. of the Mo solution. The reaction, which took about 1 hour, was conducted at a temperature of 37° ± 1° in a water bath. The pea-green precipitate was filtered off, resuspended in hot water, and again filtered. The preparation was dried at 100° overnight, and the cake pulverized and dried for another 4 hours at the same temperature. The final yield of the dried green powder was 2.3589 gm. A copper analysis by the standard thiosulfate method gave a Cu content of 30.09 per cent (29.87, 30.31; average 30.09). The Mo analysis was carried out after dissolving a small, carefully weighed quantity of the unknown in concentrated nitric acid, by measurements of its radioactivity. This gave a molybdenum analysis of

TABLE II*

Distribution of Radioactive Molybdenum in Tissues of Rats Fed Molybdenum Alone and with Catechol

	Group 1	Group 2	Group 1	Group 2
Average weight, *gm.*	155.26	181.04	155.26	181.04
Time on test, *hrs.*	25.8	25.3	25.8	25.3
Mo dose, *mg.*	26.68	26.68	26.68	26.68
Catechol dose, *mg.*		6.09		6.09
	Total per tissue		Per gm. tissue	
	γ	γ	γ	γ
Blood			16.44	27.08
Stomach	301.40 (2.79)	2671.50 (19.81)	118.52	780.00
Kidneys	24.44 (0.22)	49.08 (0.36)	15.58	31.64
Liver	89.68 (0.82)	75.01 (0.56)	10.76	11.82
Intestine	1109.00 (10.26)	1703.00 (12.62)	111.55	175.60
Bone	7.54 (0.06)	9.25 (0.07)	82.80	36.04
Heart	4.49 (0.04)	8.40 (0.06)	8.20	13.26
Lungs	12.53 (1.15)	25.30 (0.19)	12.37	21.42
Carcass	898.50 (8.11)	2219.50 (16.46)	6.90	13.40
Feces	731.00 (6.56)	998.00 (7.40)	501.00	1219.25
Urine	7930.00 (71.37)	5630.00 (41.74)		
Recovery, %	41.60	50.20		

* In this experiment one rat was fed molybdenum alone and two rats were fed molybdenum plus catechol. The figures in parentheses are the per cent of total Mo recovered.

28.51 per cent (28.00, 29.03; average 28.51). A previously prepared batch of copper molybdate in which inert Mo had been used was analyzed by the α-benzoin oxime precipitation technique (13) and gave an Mo content of 30.14 per cent (29.97, 30.30; average 30.14).

DISCUSSION

The normal Cu content of the purified ration containing 4 per cent of Salts IV as used in these experiments is 7.73 mg. per cent of Cu. The addition of 2.00 mg. per cent of Cu would provide a level of 9.73 mg. per cent of Cu, and this is apparently capable of counteracting to a large extent the toxicity of 40 mg. per cent of Mo. It would thus appear that the therapeutic effect of Cu occurs at a level too low to account for the formation of a less toxic, insoluble copper molybdate of approximately equal per cent copper and molybdenum.

On the other hand it is extremely unlikely that in the body of the rat Mo will combine only with Cu. Indeed the data in Table II suggest a slower elimination of Mo when fed with catechol. If molybdenum complexes were formed with the substances present in the alimentary canal, as suggested by the English workers, one might expect rather irregular degrees of absorption from the digestive tract. Such irregularities can be seen in the Mo analyses reported in Table I for the stomach and intestine but not for the other tissues of a more definite chemical composition.

The recovery of radioactive molybdenum was quite low. Even in those cases in which urine and feces were collected the total recovery did not exceed 50 per cent. Seepage into porcelain crucibles, adherence to metal cages, etc., are probable avenues for losses.

Whole liver substance is of such complex constitution that its powerful rôle in the counteraction of molybdenum toxicity is difficult to elucidate. The copper content of dried whole liver substance is not sufficient to account for its therapeutic effect. On the other hand the combined effect of its copper content, growth stimulation on this purified ration, and its improvement of the general nutrition of the animal might account for its antagonism to molybdenum toxicity.

SUMMARY

The toxicity of molybdenum when fed as sodium molybdate in a purified ration has been studied with rats. A level of 500 mg. per cent resulted in death after the 1st week, whereas levels of 100 and 50 mg. per cent permitted greatly reduced growth rate. In these rats there was no obvious gross pathology or change in blood picture.

The toxicity of a diet containing 40 mg. per cent of Mo could be largely overcome by feeding an additional 2 mg. per cent of Cu to a diet already

containing 7.73 mg. per cent of Cu. Other minerals such as iron, zinc, or cobalt appeared to be without effect on Mo toxicity, but whole liver substance afforded a marked degree of protection.

By means of radiomolybdenum (Mo^{99}) as a tracer the fate of a given dose of molybdenum was determined. Analysis of various tissues at 2½ hours, 1 day, and 2 days revealed most of the element to be in the stomach, intestine, feces, and urine. At the end of 2 days the kidneys and bone contained slightly higher levels per gm. than the other tissues examined. The simultaneous administration of a dose of Cu did not influence the distribution of molybdenum. However, all tissues from two rats fed a simultaneous dose of catechol contained considerably more Mo after 1 day than the control group which received only molybdenum.

A copper molybdate was prepared which assayed 30.09 per cent Cu and 28.51 per cent Mo. It is suggested that the toxicity of molybdenum may be neutralized to at least some extent through combination with certain substances, including copper, of the diet.

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A DIRECT TURBIDIMETRIC METHOD FOR DETERMINING ETHEREAL SULFATES IN URINE*

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During the course of an investigation into the excretion of "detoxication products" in the chicken (1) the need arose for a reasonably simple and reliable method for the estimation of "ethereal sulfate" in quantities of 0.1 to 1 mg. of SO_3 in volumes of some ml. of chicken urine. As none of the methods tried seemed satisfactory in the form described, a modification was developed.

It appears that in all methods hitherto described ethereal sulfate is determined as the difference between the free and total sulfate. If the procedure used for the sulfate determination is not very accurate (which is undoubtedly the case with all existing methods when the amount determined is less than 1 mg.), the error inherent in the value for ethereal sulfate may well be considerable. This is also evident from an inspection of the values given in the literature, for instance by Morgulis and Hemphill (2) and Treon and Crutchfield (3). A direct method seems to be preferable.

It has been found that free sulfate (together with interfering substances) may be precipitated with barium chloride, and the resulting excess barium by the use of sodium carbonate, without apparent loss of ethereal sulfate.

For the determination of sulfate after hydrolysis the turbidimetric method is undoubtedly the most rapid (3), and its accuracy seems quite satisfactory and at any rate not inferior to the benzidine or chromate method. Treon and Crutchfield (3) pointed out that the barium chloride should be added as a solid, not in solution. The stability and reproducibility of the cloud are then very satisfactory. These authors followed the customary practice of using a relatively large quantity of hydrochloric acid for the hydrolysis and subsequently nearly neutralized the acidity of the solution. It is, however, possible to hydrolyze the ethereal sulfates at a relatively low acidity and develop the cloud without neutralization.

Reagents—

Acid barium chloride solution. Dissolve 1 gm. of barium chloride + $2H_2O$ in water, add 2 ml. of concentrated hydrochloric acid (sp. gr. 1.19), and dilute to 100 ml.

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Sodium carbonate solution. 5 per cent (weight by volume).

Dilute hydrochloric acid. 10 ml. of concentrated hydrochloric acid diluted to 100 ml.

Barium chloride, crystalline, of uniform size, preferably 30 to 50 mesh.

Method

5 ml. of chicken urine (undiluted when fluid, or suitably diluted) are mixed with 1 ml. of acid barium chloride solution in a centrifuge tube and centrifuged at 3000 R.P.M. for about 10 minutes. The centrifugate is drained into a clean centrifuge tube and mixed with 1 ml. of sodium carbonate solution, allowed to stand some minutes, and centrifuged 10 minutes or more. 3 ml. of this centrifugate are pipetted into a test-tube, and after addition of 1 ml. of dilute hydrochloric acid the test-tube is covered with a glass ball and heated in a boiling water bath for 30 minutes. After cooling, 0.1 ± 0.005 gm. of barium chloride is added. The test-tube is gently shaken until the substance has dissolved and the solution is transferred to a cuvette. The extinction has been measured with the aid of a Weca photoelectric colorimeter (according to Havemann), the 2 cm. micro cuvette being used.

DISCUSSION

The removal of interfering substances is insured by the precipitation of free sulfate at a considerable acidity. Otherwise uric acid may interfere and cause considerable turbidity in the final stage (this seems to be due to the low solubility of the acid barium urate). This fact makes the difference method especially unsuited for chicken urine.

It is best to take chicken urine representing no more than 15 minutes excretion. Otherwise some slight turbidity may remain after the addition of sodium carbonate and following centrifugation.

The rate of hydrolysis has been tested for resorcinol sulfuric ester and phenol sulfuric ester (potassium salts were used). The results for the latter substances are as follows:

Hydrolysis time, *min.*	2	4	7	10	15	30	60	90
Phenol sulfuric ester hydrolyzed, *mg.*	0.081	0.195	0.271	0.300	0.299	0.302	0.300	0.296

Similarly resorcinol sulfuric ester is completely hydrolyzed after 15 minutes. It is obvious that the concentration of HCl (about 0.25 N) is quite sufficient for hydrolysis of this type of ethereal sulfate in less than 30 minutes. It is also clear that care must be taken to avoid prolonged standing or heating of acid solutions containing ethereal sulfates, if hydrolysis is to be avoided. This is probably the reason why treatment with perhydrol in boiling acid solution for the removal of reducing substances, as recommended by Morgulis and Hemphill (2), may result in heavy losses of ethereal sulfate.

The density and stability of the cloud at different acidities are illustrated by the figures in Table I. The density is not significantly influenced by considerable change in acidity. The stability seems to be slightly better at lower acidity. The difference does not seem to warrant the introduction of the additional operation of neutralization. The cloud appears very rapidly, except at the lowest concentrations. When the cloud is just visible, it is necessary to let the solution stand a few minutes before measuring the extinction; otherwise a considerable error may be introduced.

The relation between the extinction and the concentration is a straight line which meets the *y* axis slightly above the origin.

TABLE I

Density and Stability at Different Acidities

The samples contained 1 ml. of dilute HCl, 2 ml. of sulfate solution, and 1 ml. of sodium acetate solution.

Colorimeter readings at	Acetate concentration				
	0	0.25 N	0.50 N	0.75 N	1.0 N
min.					
5	160	158	155	159	155
20	144	150	148	149	148
60	133	140	142	140	143

The standard deviation of a single determination is about 0.007 mg. of SO_3 (calculated from eighteen pairs of duplicate determinations), and the scatter seems to be fairly independent of the concentration. Thus the relative accuracy increases with the concentration.

The recovery of phenol or resorcinol sulfuric esters added to chicken urine is essentially complete (average recovery 97 per cent).

No light filters have been used in the present study for practical reasons connected with the particular apparatus used. The extinction is, however, higher when blue light is used.

The method seems to be applicable to other urines as well as to chicken urine. It is then, of course, necessary to measure the extinction of the urine before the final addition of barium chloride, and subtract the value from the final extinction. Satisfactory results have been obtained with rabbit urine.

SUMMARY

A turbidimetric method for the estimation of ethereal sulfate in urine is described. Free sulfate and interfering substances are removed by barium

chloride and the excess barium by sodium carbonate. Hydrolysis is the effected in 30 minutes in approximately 0.25 N hydrochloric acid at 100 The resulting sulfate is then determined turbidimetrically after precipi tation as barium sulfate in the unneutralized solution. It is possible t determine 0.1 to 1.0 mg. of SO_3 in 5 ml. samples. The standard deviation o a single determination is about 0.007 mg. of SO_3.

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ISOLATION AND CHARACTERIZATION OF CHLOROMYCETIN

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A new crystalline antibiotic, obtained from filtrates of submerged aerated cultures of a *Streptomyces* sp., for which the name *Chloromycetin* was proposed, has been described in a preliminary note. It contained both nitrogen and non-ionic chlorine and appeared to be a neutral compound. In addition to a marked bacteriostatic activity in broth-dilution assays against several Gram-negative bacteria, it showed marked chemotherapeutic activity against *Rickettsia prowazeki* in screening tests with chick embryos (1) and against a number of rickettsiae and one virus when tested in embryonated eggs or in mice (2). Furthermore, it had a low order of toxicity in experimental animals and appeared to be well absorbed when administered orally to mice and dogs (1). It is the purpose of this paper to record pertinent observations made prior to the isolation, to give specific examples of two different methods of isolation, and to describe certain properties of the crystalline compound.

EXPERIMENTAL

Assay—The antibiotic activity was followed by means of a turbidimetric method, described by Joslyn and Galbraith (3), with *Shigella paradysenteriae* (Sonne) as the test organism. As soon as crystalline Chloromycetin became available, potencies were estimated in terms of weight of pure antibiotic from a standard curve.

Stability—The antibiotic was found to be quite stable in the crude culture (pH 6.81), in the filtrate after removal of the mycelium (pH 2), and in the latter adjusted to pH 7. Within the limits of error of the assay no loss of activity was apparent when these solutions were stored at 5° for a month. Thus Chloromycetin was found to be much more stable than penicillin and further experiments demonstrated it to be more stable than streptomycin in the acid range. A sample of a crude filtrate in normal hydrochloric acid retained all of its activity for 24 hours at 25° (see Table I), whereas under similar conditions streptomycin was about 90 per cent destroyed. On the other hand, both are unstable in dilute alkali, Chloromycetin being more than 87 per cent inactivated at pH 10.82 in 24 hours at 25° (see Table I).

Extraction from Aqueous Solution with Organic Solvents—A rather extensive study of this problem was made with a total of fourteen solvents. The distribution between solvents was made at an acid, an approximately neutral, and an alkaline pH (see Table II). As a result of these studies it was concluded that a number of solvents, cyclohexanone, butanol-1, ethy acetate, methyl isobutyl ketone, nitrobenzene, nitromethane, and diethy ether, possessed highly favorable distribution coefficients throughout th pH range tested, that the distribution coefficient was approximately 1 in th case of ethylene dichloride, and that the ratio was in favor of the aqueou

TABLE I

Stability of Chloromycetin in Submerged Culture Filtrates for 24 Hours at Varie pH (25°)

pH of solution	Activity retained
	per cent
0.40	100
2.15	100
7.00	100
9.56	100
10.82	<13

TABLE II

Distribution of Chloromycetin between Equal Volumes of Organic Solvents and Wate at 25° and Different pH Values

Solvent	Per cent of total activity in solvent phase		
	pH 2.15	pH 6.50	pH 9.00
Cyclohexanone	>92	>92	>92
Butanol-1	>92	>92	>92
Ethyl acetate	>92	>92	>92
Methyl isobutyl ketone	>92	>92	>92
Nitrobenzene	>92	62	83
Nitromethane	83	83	75
Diethyl ether	75	75	75
Ethylene dichloride	50	50	50
Chloroform	37	25	25
Carbon tetrachloride	25	12	50
Trichloroethylene	0	12	25
Benzene	25	12	0
Dichloroethyl ether	0	0	25
Petroleum ether	0	0	12

phase in the case of chloroform, carbon tetrachloride, trichloroethylene benzene, dichloroethyl ether, and petroleum ether. The data also indicate that the antibiotic was essentially a neutral compound.

Adsorption and Elution—Concurrent with the distribution experiment a study of the behavior of Chloromycetin toward certain common adsorbin agents was made. It was found that Chloromycetin was not appreciabl adsorbed from either acidified or neutral aqueous solutions by 2.5 per cent Super Filtrol; on the other hand it was readily adsorbed by 1 per cent Nuchar C-190-N at either an acid or neutral pH. Preliminary experiments indicated that at least 40 per cent of the antibiotic could be eluted with diethyl ether or 80 per cent acetone. Other experiments demonstrated that some purification could be effected by filtering an ether solution through a column of aluminum oxide which had previously been adjusted to pH 4.7 and dried at 100°. Under suitable conditions the antibiotic was recovered in the filtrate and some highly pigmented material remained on the column.

Isolation—With the foregoing facts procedures were devised for the concentration, purification, and isolation of the antibiotic, two of which are illustrated by describing typical experiments.

Procedure A—To 12 liters of the crude culture (pH 7.39), containing 50 γ per ml. of antibiotic (total 600 mg.), were added 190 ml. of 3 N hydrochloric acid to adjust the pH to 2. 304 gm. of Super Filtrol were added and the suspension stirred for 30 to 60 minutes. After adding 304 gm. of Hyflo Super-Cel, the solution was filtered by suction through a 50 gm. pad of Hyflo Super-Cel. The filter cake was washed with about 1200 ml. of distilled water and discarded. The combined filtrates and washings, which contained 526.5 mg. of Chloromycetin, were extracted by stirring for 10 minutes with 0.25 volume of ethyl acetate. After removing the ethyl acetate layer, the extraction was repeated once. The extracted aqueous layer, which contained virtually no antibiotic activity, was discarded and the combined ethyl acetate extracts were dried over 50 gm. of anhydrous sodium sulfate at 5°.

After the sodium sulfate was removed by filtration, the ethyl acetate was removed *in vacuo* at a bath temperature of 30°. The activity was extracted from the brown residue with small quantities of diethyl ether, the total volume of extract being 300 ml. The ether extract contained 504 mg. of the antibiotic or 84.0 per cent of the amount in the crude culture.

The ether extract was divided into three parts and each part poured through a 15 gm. column ($\frac{3}{4} \times 6.5$ inches) of aluminum oxide (Brockmann) which had previously been adjusted to pH 4.7 with hydrochloric acid. 120 ml. of diethyl ether, in 20 ml. portions, were percolated through each column. The receivers were then changed and similar quantities of ether were used in order to collect a total of five fractions from each column. Considerable quantities of brown material remained on the columns, while the antibiotic was found in the filtrate fractions. The first fractions from the three columns were pooled and found by assay to contain 418 mg. of antibiotic; the pooled second fractions contained 65 mg. and the pooled remaining fractions 17 mg., or a total of 500 mg. was found in all the ether fractions.

The first and second pooled fractions from the three columns described above, containing 483 mg. of antibiotic, were combined with similar fractions from another lot, containing 79 mg. of antibiotic. The ether was removed *in vacuo* and the residue extracted repeatedly with distilled water, the total volume being 625 ml. The combined aqueous extracts were then extracted twice with 0.5 volume of petroleum ether. Considerable brown inert material was removed by the latter solvent. This was discarded. The water phase, containing the antibiotic, was concentrated *in vacuo* at a bath temperature of 30° to a volume of 23 ml. During the course of the concentration the antibiotic crystallized. After storing the concentrate at 5° overnight the crystals were removed by filtration, washed three times with 1.7 ml. of cold distilled water, and dried *in vacuo* over calcium chloride. Yield 440 mg., melting at 146.7–147.7° (corrected). After recrystallization from methylene dichloride, ethylene dichloride, and a mixture of diethyl ether and petroleum ether the melting point remained constant at 149.7–150.7° (corrected). A further quantity of the antibiotic was recovered from the aqueous mother liquors by repeated extraction with diethyl ether, removing the ether *in vacuo*, extracting the residue repeatedly with boiling methylene dichloride, and concentrating the solution until crystals began to appear. After storage for several days at −5° the solvent was removed by decantation and the antibiotic recrystallized from ethylene dichloride. Yield 50 mg., softening at 147.7° and melting at 148.7–150.7° (corrected).

Chloromycetin was originally isolated from crude cultures containing about 20 γ per ml. by means of this procedure. As the potencies of the crude cultures increased and as changes in the composition of the fermentation medium were made, it was possible to adopt simpler procedures. The following is illustrative of the changes made.

Procedure B—The crude culture was treated with Super Filtrol and Hyflo Super-Cel as in Procedure A, except that a pH adjustment was not made. Immediately prior to extraction the pooled filtrates and washings were adjusted to pH 8.5 to 9.0 with sodium hydroxide and extracted twice with 0.25 volume of ethyl acetate. The extracts were concentrated to contain about 20 mg. of Chloromycetin per ml. To 80 ml., containing 1.650 gm., were added 20 ml. of kerosene. The solution was washed twice with 10 ml. of 0.01 N sulfuric acid, once with 10 ml. of 5 per cent sodium bicarbonate, and twice with 10 ml. of distilled water. After drying over 3 gm. of anhydrous sodium sulfate and filtering and washing the salt with anhydrous ethyl acetate, the solvent was removed partially by distillation *in vacuo* at 37°. After standing overnight at 5° the crystals were removed by filtration and digested repeatedly with 10 ml. portions of petroleum ether until the supernatants were colorless. After drying *in vacuo* 1.385 gm. were obtained, melting at 148.5–149.5° (corrected). On recrystallization from 45 ml. of ethylene dichloride, to which 50 mg. of Nuchar C-190-N had been added, 1.168 gm. were obtained, melting at 149.2–150.2° (corrected).

Properties—Chloromycetin usually crystallizes from water or ethylene dichloride as colorless needles or elongated plates (see Fig. 1). Some batches of crystals have been light yellow and this color was removed with difficulty, if at all. After several recrystallizations from different solvents it has a constant melting point of 149.7–150.7° (corrected). It is optically active, $[\alpha]_D^{25} = -25.5°$ (ethyl acetate), and has a characteristic absorption spectrum in water or 0.1 N hydrochloric acid with a single maximum at λ 278 mμ. Analysis, C 41.11, H 3.89, N 8.60, Cl (nonionic) 21.71. The solubility in water, 70 per cent aqueous propylene glycol, and propylene glycol (25°) is about 2.5, 60.6, and 150.8 mg. per ml. respectively. Chloromycetin is very soluble in methanol, ethanol, butanol-1, ethyl acetate, and acetone, fairly soluble in diethyl ether, and insoluble in benzene and petroleum ether. It is relatively insoluble in aqueous 5 per cent sodium bicarbonate and slowly soluble in cold 5 per cent sodium hydroxide. However, it produces no coloration with ferric chloride, nor does it give the shift in absorption spectrum which is characteristic of phenols when treated with alkali. The Sakaguchi, Molisch, and biuret tests are negative. Chloromycetin causes no reduction in 2 minutes in boiling Benedict's solution.

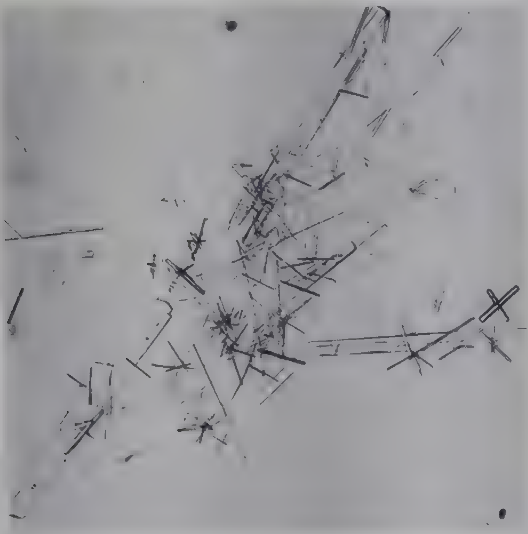

FIG. 1. Photomicrograph of crystals of Chloromycetin (from ethylene dichloride); × 150.

A solution, containing 0.2 γ per ml., caused a 50 per cent inhibition in the growth of *Shigella paradysenteriae* (Sonne). Under the same conditions 10 γ of streptomycin base were required to produce this same effect.

As was expected from the preliminary work carried out on the stability of the antibiotic in crude cultures, the pure crystalline material is quite stable in aqueous solution. No loss in activity was noted when solutions in distilled water were heated to 37° for a month or to 100° for 5 hours.

The author wishes to express his appreciation to Dr. John Ehrlich and Mr. Robert M. Smith who supplied the crude cultures used in this work; to Mr. Dwight A. Joslyn and Mrs. Margaret Galbraith for performing the numerous assays required; to Dr. John M. Vandenbelt for the absorption spectra determinations; to Mr. A. W. Spang for the microanalyses; and to Mr. Quinten W. Martin for his enthusiastic technical assistance.

SUMMARY

Methods are described for the isolation of a new antibiotic, Chloromycetin, in crystalline form from filtrates of submerged aerated cultures of a *Streptomyces* sp. Some of its more characteristic properties are described.

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THE DISTRIBUTION OF CYTOCHROME *c* AND SUCCINOXIDASE ACTIVITY IN RAT LIVER FRACTIONS*

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Rat liver suspensions can be fractionated by differential centrifugation into three main portions which are morphologically and biochemically distinct (2). Previous studies on the distribution of enzymatic activities in these fractions have shown that the activities of the succinoxidase and cytochrome oxidase systems were associated with granules approximately 0.5 to 2.0 μ in diameter, which constituted the so called large granule fraction (3, 6). In the present study, the work was extended to include cytochrome *c*, a component of the succinoxidase system which limits the activity of the system in liver homogenates or extracts. The work was facilitated by the fact that cytochrome *c*, in contrast to succinoxidase and cytochrome oxidase, can be prepared in relatively pure form and can be estimated by direct quantitative techniques. Thus experiments were possible in which not only could the distribution of cytochrome *c* present in the original liver be determined but the distribution of purified cytochrome *c* added at various stages in the fractionation could also be investigated.

Materials and Methods

Determination of Succinoxidase Activity—In tissue suspensions, the concentration of cytochrome *c* limits the activity of the succinoxidase system. In assaying for succinoxidase activity, therefore, cytochrome *c* was added in excess to insure maximum activity of the system (7).

Determinations of succinoxidase activity were also carried out without the addition of cytochrome *c*, since under certain conditions the measurement of succinoxidase activity in this manner appears to be a measure of the cytochrome *c* present. The succinoxidase activity values will be termed Q_s^{+c} in the presence of cytochrome *c* added in excess and Q_s^{-c} in the absence of added cytochrome *c*. The Q values are expressed as c.mm. of oxygen taken up per hour per mg. of dry material.

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Determination of Cytochrome c—Cytochrome *c* was estimated by the spectrophotometric method of Rosenthal and Drabkin (5).

Preparation of Rat Liver Suspensions—The liver cells were disrupted and fractionated in the centrifuge essentially as described by Claude (2). The livers from ten to twenty rats fasted for 14 to 20 hours were removed, chilled, and passed through a masher to remove connective tissue. The tissue pulp was then ground alone in a mortar for 3 minutes, and neutral water or neutral 0.85 per cent NaCl solution was added slowly to a volume corresponding to 5 times the weight of the tissue pulp.

Fractionation of Suspension of Rat Liver in Water—Removal of unbroken cells and nuclei was accomplished by two successive centrifugations of 6 and 5 minutes at 1500*g* (2). The supernatant fluid which remained after these centrifugations will be referred to as the extract, E.

The extract was centrifuged for 20 minutes at 9400*g*,[1] the supernatant, S_1, was removed, and the sediment, consisting mainly of large granules, was resuspended in a small volume of water. Portions of the large granule concentrate, Lgc, were then washed twice with either water, Lg(H_2O)w, or saline, Lg(NaCl)w, by resedimentation of the large granules from these media.

The supernatant, S_1, was recentrifuged for 15 minutes at 9400*g* to remove any unsedimented large granules. The supernatant from this centrifugation, S_2, was centrifuged 1 hour at 26,000*g*.[1] The supernatant, S_3, was saved and the sediment, consisting mainly of microsomes, was suspended in a small volume of water. Portions of the microsome concentrate, Mc, were then washed once with water, M(H_2O)w, or with saline, M(NaCl)w, by resedimentation of the microsomes from these media.

Fractionation of Suspension of Rat Liver in Saline—The removal of nuclei and of whole cells from the suspension of rat liver in isotonic saline was accomplished by three successive centrifugations of 3 minutes at 1500*g* (2).

The nuclei- and cell-free extract, E, was centrifuged for 15 minutes at 9400*g*;[1] the supernatant, S_1, was removed and the sediment consisting mainly of large granules was resuspended in a small volume of saline. Portions of the large granule concentrate, Lgc, were then washed twice with either saline, Lg(NaCl)w, or with water, Lg(H_2O)w. In addition, a portion of the granules which had been washed twice with water was washed once with saline, Lg(H_2O/NaCl)w.

It will be noted that the centrifugal force employed for the sedimentation of the large granules was considerably higher than had been used in former experiments (2). It had previously been observed that the large granules immersed in water increase in size, with a corresponding decrease in density (1, 2) and, in order to obtain adequate sedimentation of the large granules from water, it was necessary to resort to a higher centrifugal force.

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[1] The centrifuge was constructed and designed by Mr. J. Blum of the Laboratories of the Rockefeller Institute and consisted of a conical head which was rotated in a refrigerated, evacuated chamber and which supported 50 ml. lusteroid tubes at an angle of 30°.

The volumes of the original liver suspension and of each of the liver fractions were carefully determined. The values obtained were corrected for the removal of samples in the preceding steps of the fractionation procedure.

Results

The distribution of cytochrome *c* in the liver fractions was found to be profoundly affected by the medium in which the fractionation was made. The results obtained can best be presented by a description of two fractionation experiments, one of which was made in water and the other in isotonic salt solution.

Fractionation of Rat Liver in Distilled Water—The data obtained in a typical experiment in which the large granules and microsomes were isolated from a suspension of rat liver in distilled water are summarized in Table I. The volume, the dry weight, the cytochrome *c* content, and the succinoxidase activity of the different fractions are given. It will be noted that approximately 57 per cent of the dry weight of the original liver suspension was recovered in the extract, E. This would indicate that approximately 57 per cent of the liver cells had been broken. The recovery of cytochrome *c* did not, however, correlate well with the recovery of the dry weight. Thus only 40 per cent of the cytochrome *c* of the original liver suspension was recovered in the extract in contrast to the 57 per cent of the dry weight recovered in this fraction.

In confirmation of previous work (3, 6) it was found that essentially all of the succinoxidase activity present in the extract was recovered in the washed large granules. Thus 90 per cent of the succinoxidase activity present in the extract was found to be associated with the large granules washed with water, Lg(H_2O)w. Washing the granules with water or saline made little difference in the specific activity or the proportion of the total activity of this enzyme system recovered from the extract. Thus the succinoxidase Q_s^{+c} of the granules washed with water (Lg(H_2O)w) was 340 and that of the granules washed with saline (Lg(NaCl)w) was 300. In terms of total activity these values accounted for 90 and 82 per cent respectively of the total activity present in the extract.

The results obtained with cytochrome *c* were in marked contrast to those obtained with succinoxidase activity. In the first place it should be noted that only 55 per cent of the cytochrome present in the extract was recovered in the large granule concentrate, Lgc, while 96 per cent of the succinoxidase activity was recovered in this fraction. In addition 12.8 per cent of the cytochrome *c* present in the extract was recovered in the microsome concentrate, Mc. The latter finding was rather surprising in view of the fact that the other components of the succinoxidase system have not been found to be associated with the microsomes in measurable amounts (3).

TABLE I

Distribution of Cytochrome c and Succinoxidase Activity in Fractions Obtained from Suspension of Rat Liver in Water

Tissue fraction*	Volume	Dry weight			Cytochrome *c*			Succinoxidase activity		
		Per ml.	Total	Fraction of extract	Total	Fraction of extract	Per gm. dry weight	Q_s^{+c}	Fraction of extract	Q_s^{-c}
	ml.	*mg.*	*mg.*	*per cent*	γ	*per cent*	γ		*per cent*	
Liver suspension	598	54.1	32,400		23,700		732	93		
E	455	40.9	18,600	(100)	9,460	(100)	508	87	(100)	
S_1	348	30.7	10,700	57.6						
S_2	340	30.2	10,270	55.2						
S_3	319	23.5	7,500	40.3						
Lgc	125	56.1	7,020	37.8	5,200	55.0	741	224	96	12.6
Lg(H_2O)w	102	42.2	4,310	23.2	4,190	44.4	971	340	90	26.2
Washings	442	5.4	2,386	12.8						
Lg(NaCl)w	102	43.7	4,470	24.0	0	0	0	300	82	0.9
Washings	455	4.7	2,140	11.5						
Mc	58	41.2	2,400	12.9	1,204	12.8	501			
M(H_2O)w	62	31.6	1,962	10.6	802	8.5	409			
Washings	451	0.8	361	1.9						
M(NaCl)w	62	27.6	1,714	9.2	0	0	0			
Washings	443	1.0	443	2.4						

* See the text for an explanation of the symbols.

It was found that the cytochrome *c* associated with the large granule and microsome concentrates could be removed by subsequent washing with isotonic saline, although washing with water failed to remove it. As is shown in Table I, if the large granules or microsomes were washed with water (Lg(H_2O)w and M(H_2O)w), their cytochrome *c* content was 4190 and 802 γ respectively. On the other hand, the large granules and the microsomes washed with isotonic saline (Lg(NaCl)w and M(NaCl)w) did not contain measurable amounts of cytochrome *c*. Thus it is apparent that the nature of the medium used in the washing has a striking effect on the cytochrome *c* content of the large granules and microsomes.

Although a considerable amount of cytochrome *c* was associated with the large granules washed with water and the concentration of cytochrome *c* in this fraction (971 γ per gm. of dry material) was considerably higher than in the extract (508 γ per gm. of dry material), the cytochrome *c* was apparently active only to a very slight extent in the succinoxidase system. This was indicated by the low succinoxidase activity exhibited by this fraction ($Q_s^{-c} = 26$), as compared to its succinoxidase activity when cytochrome *c* was added in excess ($Q_s^{+c} = 340$).

Fractionation of Rat Liver in Isotonic Saline Solution—The results just described were in marked contrast to those obtained when the rat liver cells were disrupted and fractionated in isotonic saline (see Table II). In this case the concentration of cytochrome *c* was found to be higher in the washed large granule fractions than in the extract, with the exception of the large granules washed with water, then with isotonic saline (Lg(H_2O/NaCl)w). After the latter treatment, the large granule fraction was found to have retained only traces of its original content of cytochrome *c*. Apparently, then, the effect of water was to produce some alteration in the large granules that permitted the removal of the cytochrome *c* by the saline. Large granules washed repeatedly with saline without intermediate treatment with water retained most of their cytochrome *c*.

TABLE II

Distribution of Cytochrome c and Succinoxidase Activity in Fractions Obtained from Suspension of Rat Liver in 0.85 Per Cent NaCl

Tissue fraction*	Volume	Dry weight			Cytochrome *c*			Succinoxidase activity		
		Per ml.	Total	Fraction of extract	Total	Fraction of extract	Per gm. dry weight	Q_s^{+c}	Fraction of extract	Q_s^{-c}
	ml.	*mg.*	*mg.*	*per cent*	γ	*per cent*	γ		*per cent*	
Liver suspension	454	49.4	22,420		14,980		668	111		
E	380	35.8	13,600	(100)	8,270	(100)	608	82	(100)	
S_1	346	24.9	8,620	63.5	3,320	40.2	385			
Lgc	76	63.2	4,805	35.4	4,860	58.8	1012	232	100	149
Lg(NaCl)w	76	50.6	3,845	28.3	3,335	40.3	868	235	81	139
Washings	496	1.4	698	5.1						
Lg(H_2O)w	76	39.4	2,995	22.0	3,520	42.6	1176	310	83	21
Washings	418	4.7	1,986	14.6						
Lg(H_2O/NaCl)w	76	35.7	2,712	19.9	Traces	Traces	Traces	340	82	14
Washings	347	0.4	139	1.0						

* See the text for an explanation of the symbols.

Another striking difference between the water and saline treatments was the extremely high succinoxidase activity exhibited by the large granules washed in saline, Lg(NaCl)w. These granules had an activity which was 59 per cent as great as the activity which was exhibited when excess cytochrome *c* was added ($Q_s^{-c} = 139$, as compared to $Q_s^{+c} = 235$). On the other hand the granules washed with water had a succinoxidase activity which was only about one-seventh as great as the activity of the saline-washed granules (Q_s^{-c} of Lg(H_2O)w = 21). This difference between the succinoxidase activities of the water and the saline-washed granules was even more striking in view of the fact that the granules washed in water had a higher concentration of cytochrome *c* than did the granules washed with saline; fraction Lg(H_2O)w contained 1176 γ of cytochrome *c* per gm. of dry material, while Lg(NaCl)w contained only 868 γ.

In other experiments in which the microsomes were also isolated from saline suspensions of rat liver, another striking difference was found between the saline and the water suspensions of rat liver. No measurable amounts of cytochrome *c* were found in association with the microsomes isolated from the saline suspensions. This was in marked contrast to the considerable amounts of cytochrome *c* which had been found to be present in the microsomes isolated from suspensions of rat liver in water (Table I).

DISCUSSION

The experiments reported in this paper show that large granules sedimented from suspensions of rat liver in water or in isotonic saline and washed with water possessed a succinoxidase activity in the absence of added cytochrome *c* which was about one-seventh that of the granules maintained in saline during the entire fractionation procedure. Two main interpretations of these results can be considered, namely that the effect of water was to dilute the cytochrome *c* present in the large granules or to alter the connection of the cytochrome *c* with the other components of the succinoxidase system.

Claude (1) observed that large granules swell tremendously in distilled water and eventually disintegrate. A 2-fold increase in the diameter of the large granules would be more than sufficient to account for the low activity of the large granules washed with water, since such an increase in size would result in an 8-fold dilution of the cytochrome *c* in the large granules. The fact that both granules in saline and granules in water had approximately the same succinoxidase activity when cytochrome *c* was added in excess would also appear to support the dilution hypothesis.

The idea that the effect observed is due to dilution only is not completely satisfactory, however, in view of the fact that the cytochrome *c* could be removed from the large granules, once these granules had been treated with water.

The possibility that cytochrome *c* was present in the water-treated large granules as a result of secondary adsorption must be considered. Several observations favor such a view. In the first place, the cytochrome *c* which was present in the water-treated granules was essentially inactive in the succinoxidase system. This lack of activity might result from adsorption of the cytochrome *c* on the large granule in a location inaccessible to the other components of the succinoxidase system, rather than from dilution of the cytochrome *c* within the granules. The fact that the microsomes isolated from a suspension of rat liver in water contained cytochrome *c* while the microsomes isolated from a suspension in saline did not would seem to support the view that the effect may be due to secondary adsorption. As in the case of the large granules, it was observed that the cytochrome *c* associated with the microsomes could be removed by washing with saline. Since washing with isotonic saline did not remove the cytochrome *c* from large granules when these were originally prepared in saline, it must be concluded that the cytochrome *c* in the water-treated large granules was probably present in a different physical state.

The observation that homogenates of tissues made in isotonic media had a much higher succinoxidase activity in the absence of added cytochrome *c* than did homogenates prepared in water (4, 7) was interpreted as being due to a higher proportion of unbroken cells in the isotonic media. The ratio of succinoxidase activity in the absence of added cytochrome *c* to the activity in the presence of cytochrome *c* added in excess was thus termed the "cytolysis quotient." Since the same phenomenon was observed in the present experiments with the large granules isolated from suspensions of rat liver in water and in saline, it is obvious that cytolysis was not involved, because no cells were present in the large granule preparations. Nor is the ratio a measure of cytochrome *c* content, since the large granules prepared from a suspension of rat liver in water had a higher cytochrome *c* content than did those prepared from a suspension in saline. From these observations it can be concluded that the ratio is probably a measure of the availability of cytochrome *c* to the succinoxidase system. A more appropriate term for this ratio would be "cytochrome quotient," $Q_s^{-c}:Q_s^{+c}$.

The results which have just been described clearly show that the medium in which the fractionations of the cell are made have a profound effect upon the biochemical properties of the resultant fractions. Thus the study of the media to be used for the isolation of the components of the cell would seem to be of paramount importance if a true picture of intracellular processes is to be obtained. Together, the succinoxidase assay, the cytochrome quotient, and the quantitative determination of cytochrome *c* provide a valuable method for the studies of other media.

SUMMARY

1. The succinoxidase activity and the cytochrome *c* content of fractions of rat liver obtained by differential centrifugation were determined.

2. In confirmation of previous results, the succinoxidase activity was found to be associated almost exclusively with large granules, 0.5 to 2.0 μ in diameter.

3. Cytochrome *c* was found to be associated with the large granules and the microsomes when these were obtained from a suspension of rat liver in water; when washed with water the large granules and the microsomes retained most of the cytochrome *c* present. On the other hand, washing the large granules and the microsomes, prepared in water, with isotonic saline resulted in complete removal of the cytochrome *c*. The cytochrome *c* associated with the granules in water was not very active in the succinoxidase system, since the succinoxidase activity of the large granules was only 8 per cent of the maximum activity obtained when excess cytochrome *c* was added to the assay system, although 44 per cent of the cytochrome *c* and 90 per cent of the succinoxidase activity present in the cell-free liver extract were recovered in the large granules washed with water.

4. Cytochrome *c* was also found to be associated with the large granules sedimented from a suspension of rat liver in isotonic saline, but not with the microsomes. Most of the cytochrome *c* associated with the large granules was retained upon washing with saline or with water but was lost when the granules which had been washed with water were subsequently washed with isotonic saline. The cytochrome *c* content of the large granules accounted for 40.3 per cent, and the succinoxidase activity 81 per cent, of the respective values in the cell-free liver extract. The cytochrome *c* associated with the granules washed with saline was very active in the succinoxidase system, since the succinoxidase activity of the large granules was 60 per cent of the maximum succinoxidase activity which was obtained when cytochrome *c* was added in excess to the assay system.

5. The implications of the findings have been discussed. It is concluded that a considerable proportion of the cytochrome *c* of the liver cell is associated with the large granule fraction and is biologically active when the isolation of the large granules is made under proper conditions.

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SPECTROPHOTOMETRIC MICROMETHOD FOR THE QUANTITATIVE DETERMINATION OF THE FREE ERYTHROCYTE PROTOPORPHYRIN*

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Interest has developed recently (1–3) in the significance of the free protoporphyrin which van den Bergh and Hyman (4) reported in 1928 as being present in human erythrocytes. This "erythrocyte protoporphyrin" (EP) is identical with the protoporphyrin which, in combination with iron and globin, forms hemoglobin (1, 5). An increase in the quantity of free EP has been observed in immature erythrocytes (reticulocytes) (1, 3) in iron deficiency and in lead and gold poisoning (1) as well as in cases of anemia associated with infection (2). It has been postulated that the most common cause of increased EP is uncompleted hemoglobin synthesis, whether due to the immaturity of the red blood corpuscle or the consequence of failure to synthesize hemoglobin through lack of iron or other building stones, a fault in iron metabolism or interference with hemoglobin synthesis in some other way (3).

Van den Bergh and Hyman (4) devised a method for the quantitative determination of this porphyrin which depended on its extraction from the red blood corpuscles with a mixture of 3 volumes of ethyl acetate and 1 volume of glacial acetic acid. The concentration of the porphyrin was determined by measuring in a stufenphotometer the intensity of fluorescence exhibited under ultraviolet light by a final 5 per cent HCl solution.

Modifications of this method by van den Bergh and Grotepass (6), Lageder (7), Vigliani and Angeleri (8), Schumm (9), and Seggel (10) have been concerned mainly with the purification of the protoporphyrin extracted by van den Bergh's procedure and the measurement of the concentration of the protoporphyrin in the final HCl solution. This concentration has been determined by measuring the intensity of fluorescence or the absorption within a given spectral range.

All of these methods have one or more of the following disadvantages: too large a volume of blood required, difficulty in measuring the intensity of fluorescence by color matching in the stufenphotometer, or technical difficulties in determining the absorption in the ultraviolet, as is the case in Schumm's spectrographic method. Some of these objections were overcome in the method of Grinstein and Watson (11) who used an Evelyn photoelectric colorimeter for measuring the absorption by the protoporphyrin solution of the ultraviolet light transmitted by Filter 400. The original van den Bergh extraction procedure was reduced in this method from 2 hours to a few minutes. By this means determinations of free erythrocyte protoporphyrin in 10 ml. of blood can be made with good accuracy. The sensitivity of the Evelyn instrument is limited, however, by the much lower absorption obtained with solutions of protoporphyrin in 5 per cent HCl at the wave-length of maximum transmittance of Filter 400 and also by the wide range (380 to 420 mμ) of light transmitted by this filter. The sensitivity can be greatly increased by measuring the absorption at 407.5 mμ, which is the wave-length of maximum absorption for protoporphyrin in 5 per cent HCl (see Fig. 3, *A*). The micromethod which is described here is based on the use of the Beckman spectrophotometer. It makes possible the measurement of protoporphyrin in much smaller quantities of blood than have been used heretofore.

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† On leave from the University of Cordoba, Cordoba, Argentina.

EXPERIMENTAL

Spectrophotometric Properties of Protoporphyrin 9 (*Type III*)—The protoporphyrin 9 used in these studies was prepared from hemoglobin by a method reported recently (12). Spectrophotometric measurements were carried out with the Beckman spectrophotometer, model DU.

In Fig. 1 is shown the absorption curve of the protoporphyrin dissolved in 25 per cent HCl. The absorption intensity corresponding to the three bands can be seen. The millimolar extinction coefficient at 411 mμ (wave-length of maximum absorption in the ultraviolet band) is 17 times greater than that at 557 mμ and 47 times greater than that at 602 mμ. In Fig. 1 the transmittance curve of Filter 400 used in the Evelyn instrument is shown. It can be noted that at 398 mμ, where the transmittance of the filter is maximum and equal to 24.8, the millimolar extinction coefficient (ϵ) is reduced from a maximum of 278 at 411 mμ to 110. At 411 mμ, the wave-length of maximum absorption of protoporphyrin in 25 per cent HCl, the transmittance of the filter is reduced from 24.8 to 18.7.

In Fig. 2 calibration curves are shown for the protoporphyrin dissolved in 25 per cent HCl determined with the Beckman spectrophotometer at 411 mμ, band width of 0.6 mμ, and with the Evelyn photoelectric colorimeter, Filter 400. The data for the spectrophotometer are given in Table I. The calibration curve obtained with the spectrophotometer is a straight line, at least up to 200 γ per 100 ml. of solution; that is, the absorption follows the Lambert-Beer law. This is not true of the curve obtained with the Evelyn instrument, since the light used is not monochromatic. It

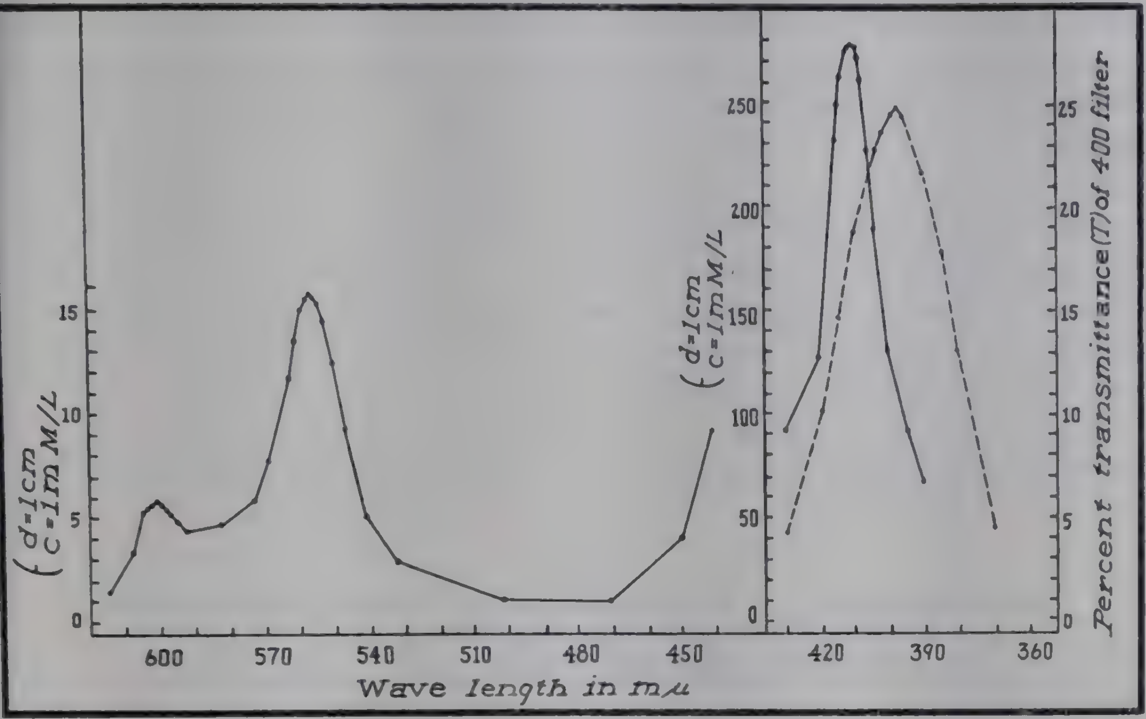

FIG. 1. Absorption curve (continuous line) of protoporphyrin 9 dissolved in 25 per cent HCl. The scale for the *millimolar extinction coefficient* (ϵ) is 10 times greater for the wave-length of 615 to 460 mμ than at lower wave-lengths. The transmittance curve of Filter 400 of the Evelyn photoelectric colorimeter is indicated by the interrupted line.

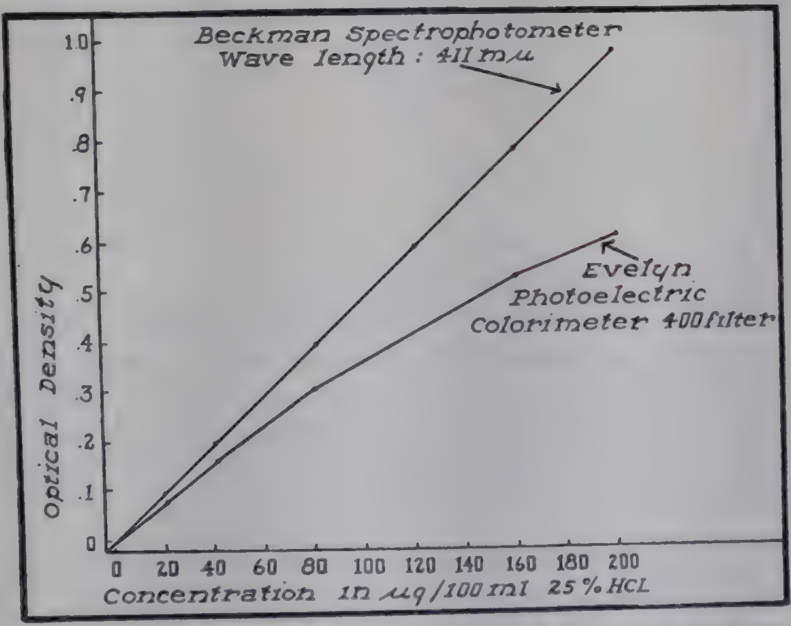

FIG. 2. Calibration curves for the protoporphyrin 9 dissolved in 25 per cent HCl

can be seen that higher optical densities were obtained with the spectrophotometer even though the measurements were made with a depth of 1 cm. of solution which is almost half of the depth used with the Evelyn photoelectric colorimeter. Considering the fact that the Beckman instrument permits measurement at the wave-length of maximum absorption, as well as the fact that this instrument requires only 3 ml. as compared to 10 ml. for the Evelyn colorimeter, the sensitivity is thus capable of being increased about 10 times. However, since the depth of the solution in the Evelyn instrument is almost double that in the Beckman, the actual sensitivity increase is only about 5 times. In general it can be stated, therefore, that quantities of protoporphyrin as low as 0.3 γ in 3 ml. of solution can be measured with good accuracy.

In Fig. 3, *A* is shown the variation of the wave-length of maximum absorption with changes in the HCl concentration from 5 to 25 per cent HCl. These observations are consistent with less complete observations by Schumm (9). Fig. 3, *B* shows that the specific extinction coefficient remains practically constant in the range of 5 to 25 per cent HCl if the measurement is made at the wave-length of maximum absorption. Fig. 3, *B* also demonstrates that if measurements are carried out at a single wave-length, for instance at 411 mμ, an error is introduced. In Fig. 3, *C* the intensity of fluorescence of the protoporphyrin in different HCl concentrations is shown. This has already been reported by Grinstein and Watson (11) for concentrations of HCl between 1 and 5 per cent. The intensity of fluorescence was measured with a Klett fluorimeter, model 2070. The sensitivity corresponds to a standard of fluorescence of 0.1 mg. of quinine sulfate in 100 ml. of 0.1 N sulfuric acid with a potentiometer setting at

TABLE I

Data for Construction of Calibration Curve for Protoporphyrin 9 Dissolved in 25 Per Cent HCl

Absorption at 411 mμ, measured with the Beckman spectrophotometer, band width of 0.6 mμ.

Protoporphyrin	Optical density, d = 1 cm.	$K = \frac{\text{Protoporphyrin concentration}}{\text{Optical density}}$
γ per cent		
20	0.097	206
40	0.196	204
80	0.395	205
120	0.587	204
160	0.783	204
200	0.978	204
Average................		204.5

300. Lamp Filter 597 and photocell Filter 306 were used for the quinine fluorescence and photocell Filter 245 for the porphyrin fluorescence. A striking decrease of the intensity of fluorescence with increase of the HCl concentration is evident.

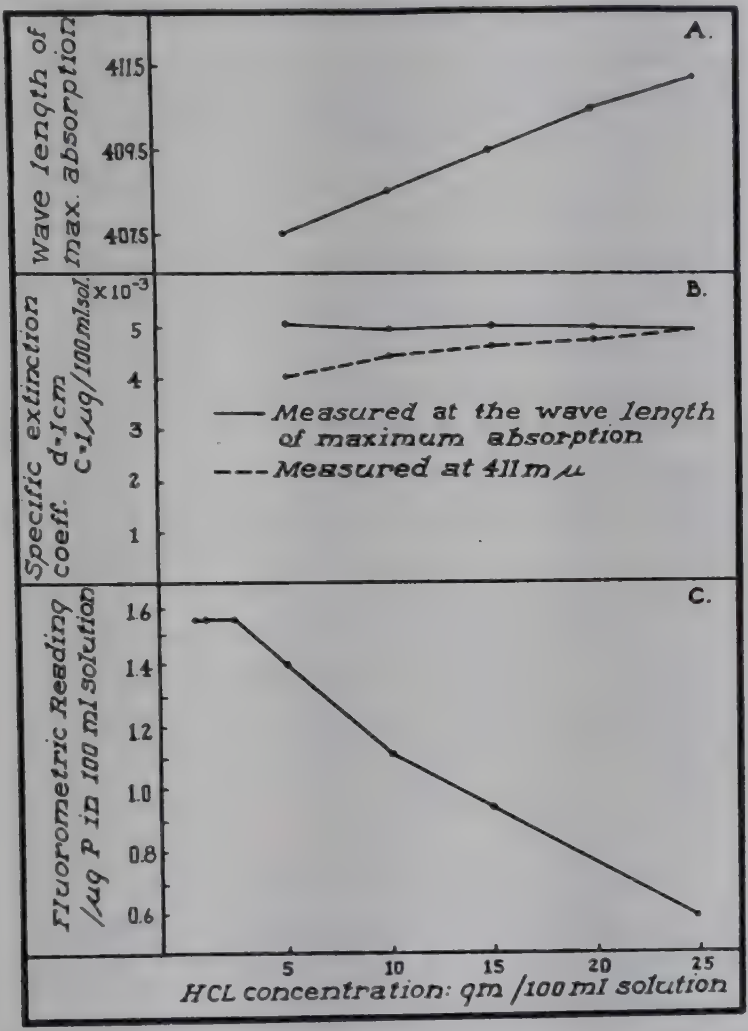

FIG. 3. *A*, variation in wave-length of maximum absorption of protoporphyrin 9 associated with changes in concentration of HCl solvent. *B*, specific extinction coefficient of protoporphyrin 9 in different concentrations of HCl when measured at the wave-length of maximum absorption compared with measurements at 411 mμ. *C*, intensity of fluorescence of protoporphyrin 9 in different concentrations of HCl.

Details of Method—The modified method is essentially the same as that described by Grinstein and Watson (11) except that only 2 ml. of whole blood are required and the final extraction is made with 25 per cent HCl instead of 5 per cent HCl. The extraction with 25 per cent HCl reduces the number of extractions necessary and consequently reduces the volume of the final solution.

2 ml. of blood are measured into a 15 ml. conical centrifuge tube which is then centrifuged and the plasma removed. In a separate sample of the blood a hematocrit determination is made. The cells are thoroughly mixed with 10 ml. of a mixture of 3 volumes of ethyl acetate and 1 volume of glacial acetic acid. This is added in successive portions and is stirred with a glass rod. It is important to use chemicals of the greatest possible purity. The mixture is allowed to stand a few minutes until the precipitate has settled. The supernatant is decanted through filter paper into a separatory funnel. A pear-shaped separatory funnel of 100 to 125 ml. is the most convenient. The precipitate is washed with three successive 5 ml. portions of the same extraction mixture and is decanted into the same separatory funnel through the same filter paper. The filtrate is washed with three successive 7 to 8 ml. portions of distilled water. The combined washings are extracted with 5 ml. of ethyl acetate to remove porphyrin that may have been washed out. This ethyl acetate is added to the main ethyl acetate solution. After complete separation of the water, the protoporphyrin is extracted repeatedly with 2 ml. portions of 10 per cent HCl until no further red fluorescence can be seen in the acid layer under the ultraviolet light.[1]

A saturated solution of sodium acetate is added until basic (red) to Congo red paper. 1 ml. of glacial acetic acid is added in order to reduce the tendency to form emulsions. The solution is extracted with successive 10 ml. portions of ethyl ether (hydrogen peroxide-free) until no red fluorescence can be seen in the ether layer under ultraviolet light. Usually two extractions are sufficient to accomplish this. The combined ether extracts are washed once with 2 ml. of a 1 per cent Na_2CO_3 solution to neutralize partially the acetic acid, and twice with 2 ml. portions of distilled water. After complete separation of the water (about 10 minutes) the ether is extracted about 1 minute with 1.5 ml. of 25 per cent HCl (25 gm. of HCl per 100 ml.). Practically all of the protoporphyrin is removed with one extraction. A second extraction with 0.3 ml. of the HCl is convenient in order to remove HCl solution of the first extraction. Ordinarily the total volume of solution obtained is 3 ml., which is sufficient for the absorption measurement with the Beckman spectrophotometer. The optical density is measured with a band width of 0.6 mμ and at a wave-length of 409 mμ, since the HCl concentration of the final protoporphyrin solution is 15 per cent (see Fig. 3, *A*). Measurements should be made within the 1st hour. For the calculation of the protoporphyrin concentration the constant $K = 204$ is used, K being the ratio of micrograms of protoporphyrin per 100 ml. of 25 per cent HCl divided by the *optical density*. For 2 ml. of blood and a final protoporphyrin solution of 3 ml. the following formula can be used for the calculation of the free protoporphyrin per 100 ml. of packed cells.

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[1] For this purpose the ultraviolet lamp of the Hanovia Chemical and Manufacturing Company, Newark, New Jersey, or G. W. Gates and Company, Franklin Square, Long Island, New York, is recommended.

$$\text{EP} = \frac{30{,}600 \times E}{Ht}$$

in which EP is the free erythrocyte protoporphyrin in micrograms per 100 ml. of packed cells, E is optical density, and Ht is the volume of packed cells per 100 ml. of blood.

TABLE II

Recovery of Protoporphyrin Added to Red Blood Cells from 2 Ml. of Whole Blood

Experiment No.	Protoporphyrin in red cells, per 100 ml. packed cells	Protoporphyrin added	Concentration found	Concentration calculated	Recovery
	γ	γ	γ *per cent*	γ *per cent*	*per cent*
1	95	0.37	128	153	84
2	87	0.52	171	190	90
3	120	0.21	144	149	96
4	120	0.42	164	178	92
5	120	0.63	186	207	90
6	120	0.84	224	236	95
7	114	0.59	172	189	91
8	114	1.18	221	264	84
9	114	1.77	290	338	86
Average...					89.8

Recovery Experiment—A solution of ethyl acetate containing a known amount of protoporphyrin was added to red blood cells and the recoveries measured. These ranged from 84 to 96 per cent (average 89.8 per cent) (Table II). In Table III are shown the recoveries obtained by adding a known amount of protoporphyrin to a mixture of ethyl acetate and glacial acetic acid such as is used in extracting protoporphyrin from the red cells. The protoporphyrin content was then measured by carrying out the various stages of the method which we have described. It can be seen that the recoveries are of the same order as those obtained when protoporphyrin was added to red cells. This indicates that the loss of protoporphyrin of approximately 10 per cent which occurred during the process of extraction (Table II) is probably due to destruction of protoporphyrin rather than to retention in the residue.

Duplicate Determinations—Table IV shows the results obtained in duplicate determinations of EP in samples of the same blood. It is seen that the differences are not greater than 4 per cent.

TABLE III

Recovery of Protoporphyrin Added to 3:1 Mixture of Ethyl Acetate and Glacial Acetic Acid

Experiment No.	Protoporphyrin added	Protoporphyrin found	Recovery
	γ	γ	*per cent*
1	0.8	0.74	93
2	1.4	1.28	91
3	1.8	1.69	94
4	2.1	1.89	90
Average..........			92.0

TABLE IV

Duplicate Determinations in Blood at Various Concentrations of Protoporphyrin

Determination No.	Protoporphyrin in blood, per 100 ml. packed cells		Difference	Determination No.	Protoporphyrin in blood, per 100 ml. packed cells		Difference
	γ	γ	*per cent*		γ	γ	*per cent*
1	62	60	3.3	9	296	295	0.3
2	103	99	4.0	10	275	271	1.5
3	162	160	1.2	11	313	304	3.0
4	167	163	2.4	12	331	328	0.9
5	176	172	2.3	13	441	424	4.0
6	188	181	3.9	14	517	502	3.0
7	258	258	0.0	15	506	500	1.2
8	239	236	1.3	16	693	686	1.0

Comment

Since this method offers good accuracy for the measurement of as little as 0.3 γ of protoporphyrin, and since normal human red blood cells contain about 30 to 35 γ per 100 ml., 2 ml. of blood are sufficient for accurate determinations when normal or increased values for EP are expected. When low values are found, or expected, a greater quantity of blood should be used.

SUMMARY

A spectrophotometric method for the measurement of free erythrocyte protoporphyrin in red blood cells is described which requires only a small amount of blood. The method depends on measurement at the wavelength of maximum absorption of a final 25 per cent HCl extract.

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THE UTILIZATION OF ADENINE FOR NUCLEIC ACID SYNTHESIS AND AS A PRECURSOR OF GUANINE*

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It is well known that organisms are able to synthesize the purines which they require for nucleic acid formation (1–4) and recent work with labeled compounds has produced evidence as to precursors which are involved. Ammonia (5), acetic acid, lactic acid, and carbon dioxide (6), formic acid (7), and glycine (6, 8) serve as biological precursors of uric acid, and presumably of the purines from which uric acid, at least in part, is derived.

Despite the fact that the organism is able to synthesize the purines which it requires, it is to be expected that purines present in the diet will be incorporated into the tissues together with those being synthesized by the body. This possibility of the incorporation of a dietary purine into the nucleoproteins was investigated by Plentl and Schoenheimer (9) who fed guanine labeled with isotopic nitrogen to rats and to pigeons. They found no incorporation of the isotope into the purines of the tissue nucleic acids but extensive conversion of the guanine into allantoin in the case of the rat and into uric acid in the case of the pigeon. Parallel experiments in which the isotopically labeled pyrimidines, uracil and thymine, were fed showed that neither of these contributed any of their nitrogen to the synthesis of nucleic acids. From these results, the authors concluded (9) that, "Neither purines nor pyrimidines supplied in the diet are utilized by the body for the synthesis of nucleoproteins," and this apparent exception to the dynamic concept of metabolism provokes speculation (10, 11).

The purine adenine is found not only in the nucleic acids but also in the adenosine triphosphate of muscle and as a structural component of certain coenzymes. In addition, adenine and its derivatives exhibit certain profound physiological and pharmacological effects not shown by guanine. It seemed possible that the metabolism of these two compounds might well be different. Therefore, it was decided to extend the observations of Plentl

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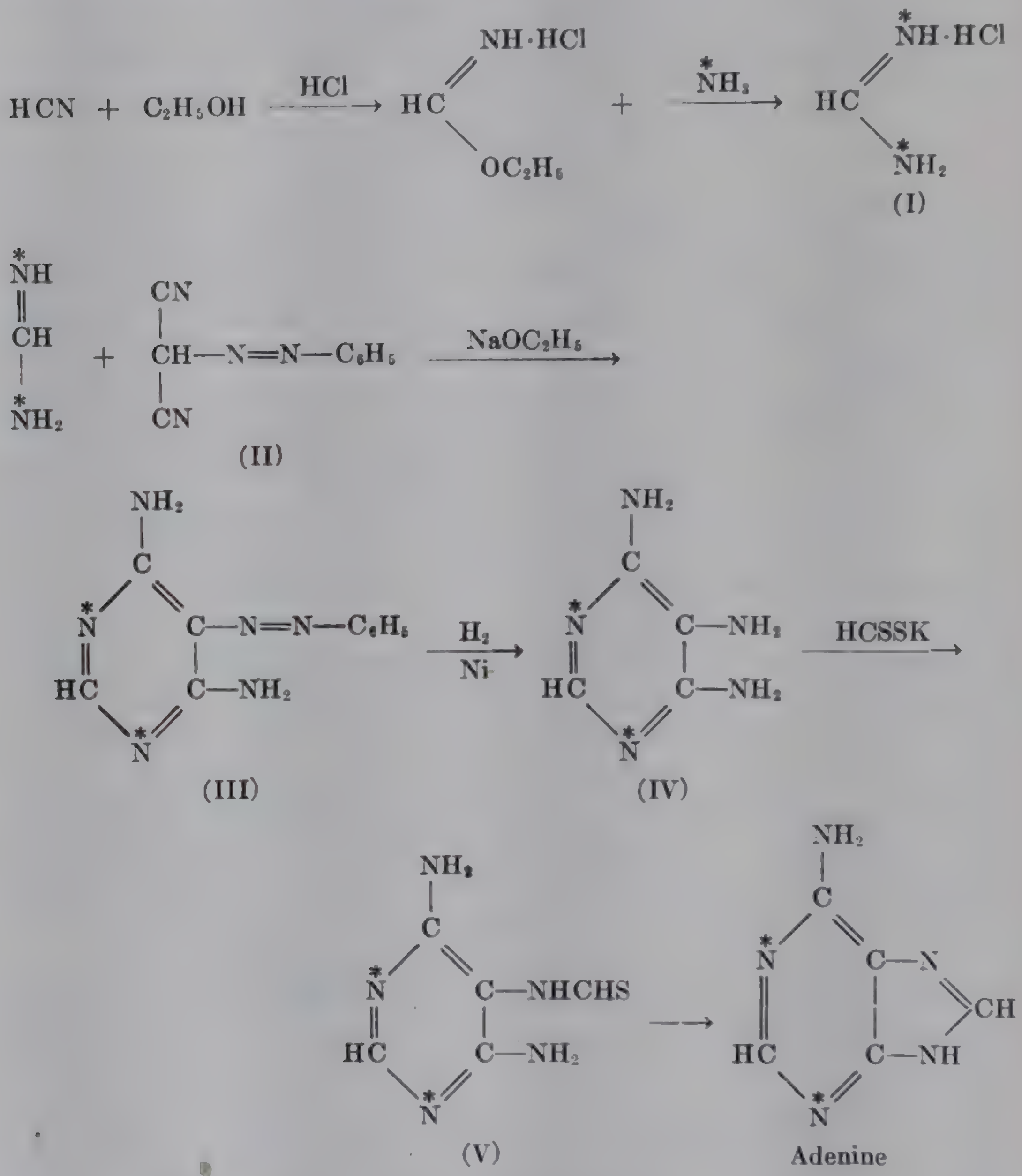
and Schoenheimer and to investigate the possibility of the incorporation of adenine into tissue constituents.

Adenine containing an excess of isotopic nitrogen in positions 1 and 3 of the purine ring was synthesized by the method of Baddiley, Lythgoe, and



Todd (12), as outlined in the accompanying scheme. This adenine was fed to adult male, Sherman strain rats. To guard against negative results due to insufficient adenine, it was desirable to administer the compound in large, but not in toxic (13), amounts. Preliminary experiments indicated that rats of this strain could be fed adenine in amounts at least as large as 250 mg. per kilo of body weight per day for a week without the appearance of any symptoms of toxicity, although with a somewhat increased water consumption. In Experiment I with isotopically labeled adenine, a total of 600 mg. of adenine per kilo of rat was administered to the animals at the rate of 200 mg. per kilo per day. In these experiments the mixed nucleic acids were isolated from the combined internal organs, with no attempt to separate the pentose and desoxypentosenucleic acids. It was found that a large amount of isotopic nitrogen of the dietary adenine had been incorporated into the nucleic acids (Table I). Degradation of these nucleic acids and separation of the adenine and the guanine revealed that not only the adenine but also the guanine contained isotopic nitrogen. The results shown in Table I indicate that in Experiment I a total of 13.7 per cent of the adenine and 8.2 per cent of the guanine of the nucleic acids was derived from the dietary adenine during the period of this experiment. On the other hand the pyrimidines, isolated as their silver salts, contained no isotopic nitrogen and were therefore not derived from the purines.

TABLE I

Feeding of Isotopic Adenine (200 Mg. per Kilo per Day)

	Atom per cent excess N^{15}	Atom per cent N^{15}, calculated on basis of 100 per cent N^{15} in adenine fed
Adenine (dietary)	6.29	100
Sodium nucleic acids	0.386	
Adenine picrate	0.536	
" (calculated from picrate)	0.857	13.7
" hydrochloride	0.850	
Guanine	0.513	8.2
Silver pyrimidines	0.00	0.0
Adenosine triphosphate	0.160	2.6
Allantoin	1.70	27.0
Ammonia	0.02	0.32
Urea	0.018	0.29

The small concentration of N^{15} found in the adenosine triphosphate (ATP) isolated from the muscle indicates that this nucleotide can also be formed from dietary adenine, although more slowly.

Allantoin, urea, and ammonia were isolated from the pooled urines. Of the allantoin excreted during the experiment, 27 per cent was derived from the adenine that had been fed. On the other hand, that there was very little degradation of the purines to either ammonia or to urea was indicated by the presence of only trace amounts of isotopic nitrogen in these substances.

This first level at which adenine was fed, 200 mg. per kilo per day, is abnormally high and may be approaching the level at which toxicities may be observed. Therefore, Experiment II was carried out in which the amount of adenine fed per day was well below the amount of purines normally metabolized by the animals. From the data of Ackroyd and Hopkins (14), it may be calculated that rats on a bread and milk diet excreted 262 mg. of allantoin per kilo per day, while Leone (15) finds 60 to 140 mg. of allantoin produced per kilo per day on diets stated to be purine-free. For Experiment II a level of 27 mg. per kilo per day was chosen as being well below the amount of purine normally turned over per day and as being equivalent, on a molar basis, to the lower level at which guanine was fed in the original experiments of Plentl and Schoenheimer (9). The crude sodium nucleates were isolated as in the first experiment and in addition copper purines were isolated directly from the dehydrated internal organs.

Table II

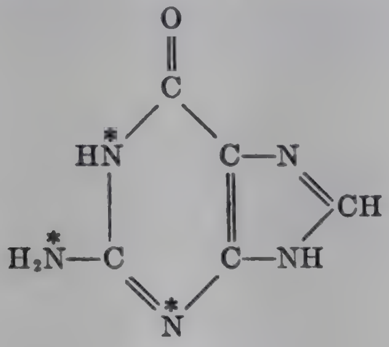
Feeding of Isotopic Adenine (27 Mg. per Kilo per Day)

	Atom per cent excess N^{15}	Atom per cent N^{15}, calculated on basis of 100 per cent N^{15} in adenine fed
Adenine (dietary)	6.29	100
Copper purines	0.23	
Purine hydrochlorides	0.23	
Adenine picrate	0.21	
" (calculated from picrate)	0.34	5.4
Guanine	0.20	3.2
Adenosine triphosphate	0.002	0.03
Allantoin	0.348	5.53
Urea	0.003	0.05
Muscle protein	0.00	0.0

The copper purines isolated from the tissues and the mixed purine hydrochlorides isolated from the crude nucleic acids contained the same atom per cent excess N^{15}. Isotope analyses (Table II) on both the adenine and guanine isolated from the mixed purine hydrochlorides showed that at this lower level there was a more efficient utilization of the dietary adenine for the formation of nucleic acids. Thus, although the amount of adenine in the diet was only 13.5 per cent of that in Experiment I, the absolute amount of isotopic nitrogen in the purines of the nucleic acids was 39 per cent of that in Experiment I. It is probably significant that in each experiment the nucleic acid adenine and the nucleic acid guanine were derived from the dietary adenine in the same ratio, that is 1.0:0.60.

At the lower level of intake the incorporation of the nitrogen of the dietary adenine into muscle ATP was negligible within experimental error, although, had the utilization for this purpose been even proportional to the amount in the diet, the isotopic nitrogen would have been easily detectable. It was also shown that the nitrogen of the muscle protein was not derived from the dietary adenine.

Because of the marked difference between these results and those of Plentl and Schoenheimer, it was decided to repeat the feeding of guanine but at a much higher level than was used by these investigators. Guanine, containing an excess of N^{15} in positions 1 and 3 of the ring and in the 2-amino group, was prepared as previously described.



Guanine

TABLE III

Feeding of Isotopic Guanine (224 Mg. per Kilo per Day)

	Atom per cent excess N^{15}	Atom per cent N^{15}, calculated on basis of 100 per cent N^{15} in guanine fed
Guanine (dietary)	6.40	100
Sodium nucleic acids	0.009	0.14
Copper purines	0.00	0.0
Allantoin	2.02	31.9
Urea	0.115	1.80

The conditions under which this guanine was fed were the same as in the adenine feeding. It was fed at a level of 327 mg. of guanine sulfate per kilo of body weight per day, which is equivalent, on a molar basis, to the higher level of adenine feeding. The results obtained (Table III) completely confirm those of Plentl and Schoenheimer. There is no evidence, even at this higher level of guanine feeding, of incorporation of dietary guanine into nucleic acids. The slight concentration of isotope found in the nucleic acids is of the order expected (5) to arise by synthesis from isotopic ammonia contributed to the body pool of ammonia from the 2-amino group of the isotopic guanine, as is the small N^{15} concentration found in the urinary urea. Just as was found by the previous investigators, a large part of the urinary allantoin arose from the dietary guanine.

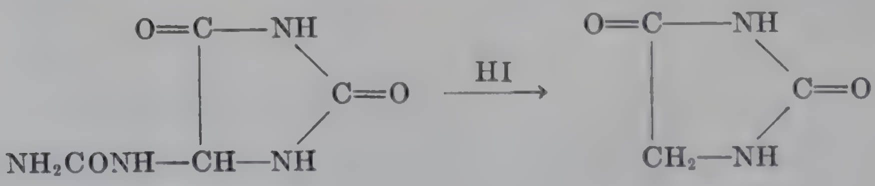
To investigate the mechanism of conversion of adenine to guanine, a sample of the guanine sulfate isolated from the nucleic acids in Experiment I was degraded. One portion of the guanine sulfate was deaminated to xanthine. Another part was oxidized by potassium chlorate to guanidine, which was isolated as the picrate. If all of the N^{15} of the guanine sulfate were in positions 1 and 3, the N^{15} concentrations of the xanthine and guanidine would be, respectively, five-fourths and five-thirds of the N^{15} concentration of the guanine. This was found to be the case (Table IV) and showed that the guanine derived from the dietary adenine still retained the isotopic nitrogen in positions 1 and 3, suggesting that the purine ring had remained intact throughout the conversion of adenine to guanine.

Table IV

Degradation of Guanine

	Atom per cent excess N^{15}	Calculated for N^{15} from positions 1 and 3 only
Guanine sulfate	0.513	
Xanthine	0.67	0.64
Guanidine picrate	0.44	
" (calculated)	0.88	0.86

The position of the isotopic nitrogen in the urinary allantoin from Experiment I was also investigated. The allantoin was degraded by reductive splitting to hydantoin (see the accompanying formulas), the isotope content of which was exactly the same as that of the allantoin from which it was derived. Thus the isotopic nitrogen, originally present in the 1 and 3 positions of the purines, had become uniformly distributed between the imidazole and urea moieties of the allantoin, and this suggests the formation of a symmetrical intermediate at some stage[1] in the degradation of the purines to allantoin.



Allantoin Hydantoin

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[1] The feeding of uric acid, labeled in the 1 and 3 positions, has also been carried out in this laboratory (42), with the finding that the isotopic nitrogen became similarly distributed in the allantoin derived from it, and it is probable that the redistribution of the nitrogens of the adenine and guanine took place at the uric acid oxidation stage.

EXPERIMENTAL

Formamidine Hydrochloride (*16*)—A 500 cc. three-necked flask, having ground glass joints, was equipped with a stirrer, with a sintered glass gas inlet tube which led to the bottom of the flask and which was interchangeable on this neck with a separatory funnel, and with a second inlet tube and a graduated separatory funnel on the third neck. This second inlet tube was attached to a system consisting of a cylinder of hydrogen cyanide,[2] followed by a flask containing a little phosphorus pentoxide, three calcium chloride tubes, and an ice-cooled spiral condenser (17). The phosphorus pentoxide-containing flask and the calcium chloride tubes were immersed in a water bath at 40°. All of the glassware was dried in the oven and assembled while hot.

The graduated separatory funnel was partially filled with ethanol freshly distilled from sodium ethylate and the second separatory funnel was filled with 200 cc. of ether freshly distilled from butyl magnesium bromide. After the apparatus had been weighed and attached to the dry hydrogen cyanide system, the main flask was surrounded by a bath at 40°. A few cc. of hydrogen cyanide were allowed to flow from the tank onto the phosphorus pentoxide and to distil from there through the calcium chloride tubes and to be condensed into the main flask. When an appropriate amount had been condensed, the apparatus was disconnected, quickly weighed, and then returned to the ice bath. The weight of hydrogen cyanide collected (about 6.25 gm.) was calculated, and the ether and slightly over 1 equivalent of ethanol were introduced (16 cc. of ethanol for 6.25 gm. of HCN). Stirring was begun and dry hydrogen chloride was introduced through the sintered glass inlet tube at a rapid rate. When the ether became saturated with hydrogen chloride and the precipitation of formimido ethyl ester hydrochloride had begun, the introduction of dry HCl was ended and the stirring and cooling were continued for 3 or 4 hours, or overnight.

The sintered glass inlet tube was then connected to a vacuum system and the ether was drawn off. A little more ether was then added through the separatory funnel and drawn off. About 100 cc. of cold dry alcohol, containing slightly less than 1 equivalent of dry isotopic ammonia (3.6 gm. for 6.25 gm. of HCN), which had been prepared (18) in the meantime, were added through the ether separatory funnel. All transfers were made with complete exclusion of moisture. The formimido ester dissolved and the crystallization of formamidine hydrochloride (I) began when the solution was cooled in a dry ice bath. The mother liquors were then removed through the sintered glass tube as before. The unchanged ammonia was recovered from the mother liquors.

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[2] American Cyanamid Company.

The latter step may also be carried out by sealing formimido ester hydrochloride and isotopic ammonia in ethanol in a glass tube and heating at 100° for 1 hour.

Isotopic Adenine—The synthesis of 4,6-diamino-5-phenylazopyrimidine (III) was carried out according to the procedure of Baddiley, Lythgoe, and Todd (12) by adding immediately phenylazomalononitrile (II) and sodium ethylate to the formamidine hydrochloride in the flask in which it was prepared. The remainder of the synthesis was carried out according to the procedures of these authors.

In the final cyclization of the 5-thioformamido-4,6-diaminopyrimidine in water as described by these authors, a pure product was not obtained. About 65 per cent of it was adenine and about 30 per cent was a more soluble compound, obtained chiefly in the second and third crops. This latter has proved to be the 5-formamido-4,6-diaminopyrimidine, which possesses an absorption spectrum almost identical with that of adenine but which may be readily distinguished from adenine by its distribution constant (K) in a butanol-1 M phosphate system, pH 6.5.[3] In this system adenine shows a K of 2.2 to 2.5, varying somewhat with concentration, while the 5-formamido-4,6-diaminopyrimidine shows a K of 0.225. The sample of adenine used in the biological experiments was recrystallized from water and was shown to be at least 98 per cent homogeneous when characterized by a twenty-four plate counter-current distribution (19, 20).

One nitrogen containing 32.0 atom per cent excess N^{15} was introduced into the formamidine used, resulting in 16.0 per cent in each of the 1 and 3 nitrogens of the purine ring of adenine, or a theoretical value of 6.40 atom per cent excess average N^{15} content for the molecule. The atom per cent excess[4] was found to be 6.29.

$C_5H_5N_5$. Calculated, N 51.9;[5] found, N 51.4

Guanidine Nitrate—Guanidine nitrate was prepared according to the procedure of Davis (21). Dicyandiamide (4.3 gm.) was fused with 9 gm. of ammonium nitrate, the ammonia of which contained 32 atom per cent excess N^{15}, and 10.8 gm. of guanidine nitrate were obtained, or 86 per cent of the theoretical yield.

Guanine Sulfate—Guanine sulfate was prepared according to the procedure of Traube (22), with the modifications of Plentl and Schoenheimer

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[3] J. F. Tinker, unpublished data.

[4] The authors wish to express their appreciation to the M. W. Kellog Company; to Mr. V. H. Dibeler and Dr. F. L. Mohler of the National Bureau of Standards; and to Mr. Steven Friedland of this laboratory, for the isotope analyses. The degree of precision possible was not always the same; as a result, some figures are quoted more precisely than others.

(9). Guanidine nitrate was treated with ethyl cyanoacetate in anhydrous methanol containing sodium methylate. The resulting 2,4-diamino-6-hydroxypyrimidine was obtained as the sulfate (58 per cent yield) and nitrosated (90 per cent yield). This derivative was reduced to the corresponding 2,4,5-triamino-6-hydroxypyrimidine compound which was obtained as the sulfate (65 per cent yield). Treatment of this triamine with formic acid yielded guanine sulfate (73 per cent yield). The over-all yield based upon the ammonium nitrate used was 21 per cent. Isotope analysis showed an atom per cent excess N^{15} of 6.40, which is equal to the expected value.

$(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$. Calculated, N 32.5;[6] found, N 32.2

Feeding Experiments—All animals used were Sherman strain rats obtained from Rockland Farms. They were kept in metabolism cages which permitted collection of urine with minimum contamination of feces. Daily quotas of food were offered them each afternoon. The rats were maintained on an unsupplemented diet for 4 days immediately prior to the experiments. The adenine as the hydrochloride and the guanine as the sulfate were mixed with pulverized and moistened Rockland rat diet (complete), the stock diet to which the rats were accustomed. In all experiments the supplemented food was fed over a 3 day period. On the 4th day the animals were given normal food, and were sacrificed at the end of that day. The urine voided during these 4 days was combined. During the process of collection it was diluted with about an equal volume of water. The rats were anesthetized by an intraperitoneal injection of 1 cc. of 25 per cent urethan. Several small injections of 25 per cent magnesium sulfate were then given at short intervals until death resulted. The thymus, lungs, heart, liver, kidneys, spleen, testes, and small intestine were immediately removed and dropped into a dry ice-ethanol freezing mixture. The intestines were first opened lengthwise, washed, and cut into small pieces. The leg and back muscles were removed and quickly frozen on a block of dry ice.

Adenine Feeding; Experiment I—Five adult male rats with a combined weight of 1133 gm. were fed a total of 680 mg. of adenine (6.29 per cent excess N^{15}) as its hydrochloride, admixed with 272 gm. of food over a 3 day period. These animals gained a total of 147 gm. during the 7 day period and in succeeding experiments the food intake was reduced from 80 to 60 gm. per kilo of body weight per day.

Adenine Feeding; Experiment II—Two adult male rats weighing a total of 602 gm. were fed 48 mg. of adenine (6.29 per cent excess N^{15}), admixed with 145 gm. of food, over a period of 3 days. Their weight remained constant.

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[6] Corrected for content of isotopic nitrogen.

Guanine Feeding; Experiment III—Four adult male rats weighing a total of 1162 gm. were fed 780 mg. of guanine (6.40 per cent excess N^{15}) as its sulfate, admixed with 210 gm. of food, over a 3 day period. Their weight remained constant.

Isolation of Nucleic Acids—The mixed internal organs, while still frozen, were homogenized with ethanol in a Waring blendor. The mixture was filtered and the residue was suspended in hot ethanol. This was repeated once more with ethanol and once with ether. The final residue was a white powder. From 16 to 20 gm. of dehydrated tissue were obtained per kilo of rat.

The sodium salts of the mixed nucleic acids were extracted by 10 per cent sodium chloride solution and the free nucleic acids were prepared (9). An average yield of 0.8 gm. of nucleic acids was obtained from 20 gm. of dehydrated viscera.

Isolation of Purines and Pyrimidines—The nucleic acids were hydrolyzed and the purines were isolated by the method of Levene (23). A total of 101 mg. of crude guanine, which yielded 78 mg. of pure guanine sulfate, was obtained in Experiment I.

$(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$. Calculated, N 32.1; found, Experiment I, N 32.3

The adenine was isolated as the picrate and the product was recrystallized three times. In Experiment I 140 mg. were obtained.

$C_5H_5N_5 \cdot C_6H_3O_7N_3 \cdot H_2O$. Calculated, N 29.3; found, N 29.1

The adenine picrate[6] was characterized by counter-current distribution analysis and absorption spectrum and was furthermore shown to be free of guanine. Similarly, the guanine ($K = 0.48$) was shown to be free of adenine.

The adenine picrate was also dissolved in dilute hydrochloric acid, extracted with ether, and adenine hydrochloride was isolated after concentration of the solution.

The filtrate from the precipitation of the mixed purine hydrochlorides was used for the preparation of silver pyrimidines. It was evaporated to a syrup, *in vacuo*, and 6 cc. of 6 N HCl were added per gm. of free nucleic acid. The solution was heated in a bomb tube at 180° for 3 hours and the residue was removed by filtration. From the solution the silver pyrimidines were prepared by the method of Kossel as described by Levene (24).

In Experiment II copper purines were also isolated directly from a portion of the dehydrated viscera according to the procedure of Graff and Maculla (25).

Isolation of Barium Salt of ATP—The frozen muscle was used for the isolation of the barium salt of ATP according to the procedure of LePage (26). An average yield of 130 mg. from 100 gm. of muscle was obtained.

$C_{10}H_{16}O_{13}N_5P_3Ba_2 \cdot 4H_2O$. Calculated. N 8.2, P 10.9
Found. Experiment I, N 8.4, P 10.0
" " II, " 7.7, " 9.5

The analytical data for Experiment I indicate a nitrogen to phosphorus mole ratio of 5.0:2.7, or a product representing 70 per cent ATP and 30 per cent adenosine diphosphate. For Experiment II the N:P ratio is 5.0:2.8.

In preliminary experiments magnesium sulfate anesthesia did not quiet the rats satisfactorily and low yields of ATP were obtained. With the preliminary urethan anesthesia, followed by magnesium sulfate, the animals succumbed without convulsions and excellent yields of ATP were obtained.

The muscle residue, after the extraction of the ATP by cold trichloroacetic acid, was used for the preparation of nucleic acid-free protein. A portion of the residue was extracted by boiling with 5 per cent trichloroacetic acid for 30 minutes. The solid was recovered by centrifugation and the extraction was repeated. The nitrogen of this residue was obtained by Kjeldahl digestion and was used for the determination of the N^{15} content of muscle protein.

Isolation of Allantoin—Allantoin was isolated from urine by a combination and modification of the methods of Schaffer and Greenbaum (27) and Wiechowski (28). 50 cc. of diluted urine were placed in a 250 cc. centrifuge tube and 35 cc. of 35 per cent phosphotungstic acid, which must be carefully purified preferably by recrystallization from ether (29), were added. The precipitate was settled by centrifugation and the supernatant was tested for complete precipitation. To the solution were then added 35 cc. of 5 per cent basic lead acetate solution, and the tube was again centrifuged and again tested for complete precipitation by this reagent. The supernatant was separated by decantation and mixed with 35 cc. of 5 per cent sulfuric acid. It was again centrifuged and the solution filtered to remove the last trace of lead sulfate. The solution was then neutralized to litmus with 5 per cent sodium hydroxide solution and the allantoin was precipitated by the addition of 100 cc. of a solution containing 1 gm. of mercuric acetate and 10 gm. of sodium acetate. After standing overnight the flocculent precipitate of mercury allantoin was collected by centrifugation and washed three times with water. The solid was dried *in vacuo* over P_2O_5. About 0.6 gm. of the dried material was usually obtained from 100 cc. of the diluted urine. The solid was ground up with water in a mortar and decomposed

[6] **Picric acid, $K = 9.25$.**

with hydrogen sulfide. After the removal of mercuric sulfide, the solution was aerated and was then concentrated *in vacuo* to about 2 to 5 cc. The allantoin, which crystallized when the solution was cooled in the refrigerator overnight, was dissolved in the minimum amount of hot water, treated with Darco, filtered, and again allowed to crystallize. The recrystallization was repeated and the prismatic crystals were dried *in vacuo*. Amounts varying from 40 to 129 mg. per 100 cc. of diluted urine were obtained. In Experiment I two portions yielded samples of allantoin amounting to 129 and 112 mg.

$C_4H_6O_3N_4$. **Calculated.** N 35.5
Found. Experiment I, N 35.7
" " II, " 35.9
" " III, " 36.0

Isolation of Urea and Ammonia—Duplicate samples of the urea were isolated as the dixanthydrol derivative as described by Fosse (30). 2 cc. of urine were dissolved in 24 cc. of water and to this solution were added 50 cc. of glacial acetic acid, followed by 5 cc. of a freshly prepared 5 per cent solution of xanthydrol in methyl alcohol. In a few minutes a silvery precipitate separated. After half an hour 10 cc. of water were added and the suspension was left overnight. The precipitate was collected and dried *in vacuo*. The dixanthydrol urea was then recrystallized three times from glacial acetic acid after treatment with charcoal.

$C_{27}H_{24}O_5N_2$. **Calculated.** N 6.7
Found. Experiment I, N 6.9
" " II, " 6.2
" " III, " 6.8

The ammonia was recovered from 5 cc. samples of the filtered urine according to the permutit method of Folin and Bell (31). The suspension of ammonia-permutit was decomposed with NaOH and the NH_3 was collected in HCl for isotope analyses.

Degradation of Guanine Isolated from Nucleic Acids—The procedure of Strecker (32) for the degradation was simplified as follows: A 41 mg. sample of guanine sulfate was suspended in 3 cc. of concentrated hydrochloric acid. To this were added in one portion 35 mg. of potassium perchlorate. The solution was stirred and let stand for 5 minutes and then placed in a water bath at 50–60° for 35 minutes, after which time all of the solid material had gone into solution. The solution was then evaporated to dryness under diminished pressure and the residue extracted with two 10 cc. portions of absolute ethanol. After filtration, the solution was evaporated to 4 cc. 3 cc. of water were added and the whole was evaporated to 1 cc. The solution was filtered and 0.5 cc. of saturated picric acid was added.

After standing overnight in the ice box 18 mg. of guanidine picrate separated. Recrystallization from water yielded 10 mg. of pure product.

$C_7H_8O_7N_6$. Calculated, N 29.1; found, N 29.0

In a pilot experiment the degradation of guanine to xanthine by the procedures described (9, 24) gave a product decomposing above 150° (guanine decomposed above 360°).

$C_5H_4O_2N_4$. Calculated, N 36.8; found, N 36.6

Degradation of 26 mg. of the guanine sulfate isolated in Experiment I yielded 13 mg. of xanthine decomposing above 150°, all of which was utilized for isotope analyses and no elementary analysis was obtained.

Degradation of Allantoin—The allantoin isolated in Experiment I was degraded to hydantoin according to the procedure described in a previous communication (33). The atom per cent excess N^{15} in the allantoin was 1.70; the hydantoin obtained from it contained 1.71 atom per cent excess.

$C_3H_4O_2N_2$. Calculated, N 28.0; found, N 28.2

DISCUSSION

As a result of these experiments demonstrating incorporation of ingested adenine into nucleic acids, the concept that dietary purines and pyrimidines in general are not precursors of the tissue nucleic acids must be modified. However, the circumstance that ingested guanine, as well as uracil and thymine (9), are not incorporated as structural components of the tissue nucleic acids still remains an anomaly.

There must be at least two pathways for the utilization of dietary adenine, since, in Experiment I, the per cent incorporation of dietary adenine into allantoin was much greater than the per cent incorporation into the nucleic acid purines. Thus, as in the case of guanine, a pathway exists for the direct oxidation of adenine to allantoin without prior incorporation into the nucleic acids.

As is shown by the results of Experiments I and II, an increase in the amount of dietary adenine available increases the incorporation of it into the purines of the nucleic acids. However, the ratio between the nucleic acid-adenine and the nucleic acid-guanine arising from ingested adenine was the same in the two experiments. Thus the step of conversion of adenine to guanine is independent of the amount of dietary adenine being utilized for nucleic acid synthesis.

The small replacement of the adenine of the ATP of muscle would appear to rule out the possibility of ATP being an intermediate in the incorporation of adenine into nucleic acids. This behavior of the adenine moiety parallels the small uptake of nitrogen from administered isotopic ammonia

(5) and the very slow exchange, with inorganic phosphate, of the phosphate esterified with the ribose (34, 35). It may be that the disproportionately greater incorporation of adenine into ATP when the larger amount was present in the diet represents an alteration of a normally very slow turnover of the ATP skeleton and that this phenomenon is related to the toxicity of large doses of adenine.

The finding that adenine is transformed into guanine necessitates a new appraisal of the relative rôles of the two purines. Perhaps only adenine is synthesized *in vivo* from smaller precursors and all other purines are derived from adenine. In connection with this it is interesting to note that among x-ray-induced mutants of *Neurospora crassa* forty-five *adenineless* mutants but no *guanineless* mutants have been detected (36), although one *guanineless* mutant of the mold *Ophiostoma multiannulatum* has been obtained (37). If the synthesis of adenine from various small precursors involves many stages while the conversion of adenine to guanine requires few, the opportunity to destroy a gene controlling a step in the synthesis of adenine is much greater.

The conversion of adenine to guanine with the retention of the isotopic nitrogen in the 1 and 3 positions of the purine ring implies retention of the intact purine ring and changes only in the substituents in the 2 and 6 positions. If free purines are involved in this transformation of adenine to nucleic acid guanine, guanine itself cannot be an intermediate and likewise xanthine, which is presumably an intermediate in the *in vivo* oxidation of the ingested guanine to allantoin, can be eliminated as one of the possible intermediates.

On the other hand it is quite possible that the conversion of adenine to guanine involves derivatives of the two purines; for instance, it may be that adenine is first transformed into its nucleoside or nucleotide, which is then converted to the corresponding guanine derivative and both may be used for nucleic acid synthesis. Adenosine so formed would also be a logical precursor for the synthesis of ATP. If labeled guanosine were available, it would be interesting to determine whether this compound would enter into the structure of the nucleic acids, and experiments on the synthesis of labeled guanosine are under way at this time.

It is conceivable, however, that adenine in the nucleic acid molecule may be transformed *in situ* into guanine and that guanine, when liberated from the nucleic acid may only be degraded to its end-products. Recent emphasis (38–40) upon possible deviations from the occurrence of the two purines in a 1:1 ratio in nucleic acids and the demonstration (41) of a phosphorylase capable of the direct removal of guanine from a nucleic acid are in accord with such a thought.

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SUMMARY

Adenine containing an excess of isotopic nitrogen in positions 1 and 3 of the purine ring has been synthesized.

The incorporation of ingested adenine into the tissue nucleic acids and to a smaller extent into the adenosine triphosphate of muscle has been shown to occur in the rat.

The rôle of adenine as a metabolic precursor of nucleic acid guanine has been demonstrated.

The observation that dietary guanine is not at all utilized for nucleic acid synthesis has been confirmed.

Evidence that adenine and guanine, either free or as components of nucleic acids, are not precursors of pyrimidines was obtained.

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FURTHER STUDIES ON THE METABOLISM OF TRYPTOPHAN AND NICOTINIC ACID BY THE RAT AND OTHER ANIMALS*

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Several reports are available which show that when relatively large amounts of L- or DL-tryptophan are fed to animals a marked rise in the urinary excretion of nicotinic acid, N^1-methylnicotinamide, or both occurs (1–8). Other work with rats and chicks fed diets containing corn, gelatin, or other tryptophan-deficient proteins and low in nicotinic acid suggested that components of the diet other than tryptophan may markedly influence the rate of growth (8–11). Specifically the type of protein and carbohydrate in the diet apparently alter the ability of the animal to utilize the dietary tryptophan efficiently as a source of nicotinic acid or affect the intestinal synthesis of nicotinic acid. Further, recent studies have revealed that vitamin B_6-deficient animals cannot effectively convert tryptophan to nicotinic acid and its derivatives (12, 13). The present study was conducted to obtain further information on the interrelationship of tryptophan and nicotinic acid, with the main emphasis on the effect of the level and kind of protein and of carbohydrate on the rate of growth and urinary excretion of nicotinic acid and N^1-methylnicotinamide.

EXPERIMENTAL

Studies with Rats—Weanling albino rats were used and fed the experimental diets for a period of 4 weeks. The animals were housed in single cages and food and water were supplied *ad libitum.* Urinary collections were made during the 3rd to 4th weeks for successive 2 or 3 day periods. The urine from two rats fed the same ration was pooled for each period of collection. Within any one experimental series, therefore, collections were obtained from at least four rats which comprised two pooled collections. In all cases collections were made from the experimental groups in each series at the same time in order to obtain data for comparable periods for all groups. The methods of analysis to determine the urinary excretion of nicotinic acid, N^1-methylnicotinamide, and tryptophan were the same as were described in previous work (4, 12).

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The basal diet used was composed of (in per cent) purified casein 12, Salts IV (14) 4, corn oil 4.7, fortified cod liver oil 0.3, cystine 0.3, sucrose 78.7, and the following B vitamins per 100 gm. of ration: thiamine 250 γ, riboflavin 300 γ, pyridoxine 250 γ, calcium pantothenate 2 mg., choline 100 mg., inositol 100 mg., biotin 10 γ, and synthetic pteroylglutamic acid (folic acid) 200 γ. Control groups were fed in addition 1 mg. of nicotinic acid per 100 gm. The effect of variations in the protein level or kind of carbohydrate in the basal or control diets on the growth rate and urinary excretion was studied and in each case the substitutions in the diet were made for an equal weight of sucrose. The effect of feeding diets containing 12 per cent casein, 12 per cent casein and 12 per cent gelatin, 18 per cent casein, or 24 per cent casein with either sucrose or cerelose as the carbohydrate and with and without nicotinic acid on the rate of growth is shown in Table I. All experiments were repeated at least once and in most cases twice. Four rats were used in each group in each series and litter mates were distributed as equally as possible among the experimental groups. From the data presented in Table I, it can be seen that the growth rate of rats increased progressively when 12, 18, and 24 per cent casein was fed with sucrose as the carbohydrate and no nicotinic acid added. The growth rates were considerably more rapid, however, when cerelose was fed instead of sucrose with 12 or 18 per cent casein. The growth rates for rats fed sucrose or cerelose with 24 per cent casein were essentially the same. The addition of nicotinic acid increased the rate of growth with either cerelose or sucrose when 12 per cent casein was fed, only slightly with sucrose when 18 per cent casein was fed, and not at all when cerelose was fed with 18 per cent casein or when cerelose or sucrose was fed with 24 per cent casein. The difference noted between the groups fed cerelose and

TABLE I

Effect of Amount and Kind of Protein and Carbohydrate in Diet on Growth Rate of Rats Receiving Nicotinic Acid-Deficient or Supplemented Diets

Protein source	Growth rate, gm. per wk.*			
	Nicotinic acid added			
	None		1 mg. per 100 gm.	
	Sucrose	Cerelose	Sucrose	Cerelose
12% casein	9 (12)	23 (8)	21 (12)	30 (8)
18% "	16 (12)	33 (12)	23 (12)	31 (12)
24% "	30 (8)	33 (8)	33 (8)	34 (8)
12% " + 12% gelatin	6 (12)	8 (8)	23 (12)	28 (8)

* The number of rats in each group is given in parentheses.

sucrose and 12 per cent casein is as great as has been observed with the use of these carbohydrates in rations containing large amounts of corn (10).

The rats fed 12 per cent casein and 12 per cent gelatin with either cerelose or sucrose grew at a slow rate unless nicotinic acid was added. Although the rats fed cerelose were somewhat superior in appearance to those fed sucrose, the difference in appearance as well as in the rates of growth was not marked. Several of the animals fed these diets exhibited a brownish red exudate particularly around the head, similar to that observed for animals fed rations high in corn or tryptophan-deficient proteins (8, 10). It is apparent, therefore, that the addition of gelatin as compared to an equivalent amount of carbohydrate increased the dietary requirement for nicotinic acid even when the tryptophan level was the same in both diets (12 per cent casein and 12 per cent gelatin as compared to 12 per cent casein).

TABLE II

Effect of Amount and Kind of Protein and Carbohydrate in Diets Devoid of Added Nicotinic Acid on Metabolism of Nicotinic Acid by Rats

Protein source	Urinary excretion of nicotinic acid*		Urinary excretion of N^1-methylnicotinamide*	
	Sucrose	Cerelose	Sucrose	Cerelose
12% casein	7.6 (8)	18.4 (8)	24.3 (8)	32.6 (8)
18% "	9.3 (9)	21.6 (8)	31.0 (9)	160 (8)
24% "	15.9 (11)	30.8 (5)	156 (12)	562 (6)
12% " + 12% gelatin	7.3 (7)	13.2 (6)	27.4 (7)	34.3 (8)

* Values expressed as micrograms excreted per rat per day. The number of determinations is given in parentheses. Each determination was made on the pooled collections from two rats for a 2 or 3 day period (see the text).

Data obtained on the amounts of nicotinic acid and N^1-methylnicotinamide excreted in the urine are presented in Table II. A progressive increase in the amounts of both compounds excreted was observed as the casein level was increased. The greatest increase was observed when the casein level was increased from 18 to 24 per cent. It should be pointed out that no nicotinic acid was added to any of these diets. Considerable variation was observed, particularly in the amounts of N^1-methylnicotinamide excreted for the different series of experiments carried out. However, collections were made simultaneously for the various groups that were compared (Table II) and the relative values are assumed to be valid. For brevity all values obtained have been averaged. The growth rates of the rats fed those diets which are limiting in nicotinic acid correlate well with the magnitude of the values for the urinary nicotinic acid compounds.

Similar results have been recently reported with the use of sucrose diets and varying levels of casein (8).

These data are further evidence that the nicotinic acid nutrition of the animal is dependent on the protein (tryptophan) intake. The kind of carbohydrate also influences the nicotinic acid nutrition of the animal, since a marked increase in the amounts of N^1-methylnicotinamide and nicotinic acid excreted was observed when cerelose was used as the carbohydrate as compared to sucrose. This was particularly apparent for the groups fed either 18 or 24 per cent casein. When additional gelatin was fed, no appreciable decrease was noted in the urinary excretion of nicotinic acid or its methylated derivative as compared to when 12 per cent casein was fed, although when gelatin was fed a depression in the growth rate was observed. Other workers (10, 11) have shown that glycine inhibits the rate of growth of rats and chicks fed diets low in nicotinic acid. Since gelatin contains large amounts of glycine, it suggests that the glycine contributed by the gelatin is one of the causative factors. Glycine and presumably other amino acids may require more nicotinic acid for metabolism than an equivalent amount of carbohydrate. The metabolic mechanism by which this probable increase in the requirement is brought about has not been determined.

To test the specificity of feeding tryptophan on the urinary excretion of nicotinic acid and N^1-methylnicotinamide, the effect of feeding related compounds was investigated. No increase in the amounts of N^1-methylnicotinamide or nicotinic acid excreted was observed when 100 mg. of tyrosine, 100 mg. of glutamic acid, and 200 mg. of DL-phenylalanine were fed per day. A slight rise in the excretion of N^1-methylnicotinamide was noted when indole was fed, but no increase was noted in the amount of nicotinic acid excreted. The slight increase in urinary N^1-methylnicotinamide excretion was apparently due to interference of indole with the method for determining N^1-methylnicotinamide (15). It appears, therefore, that of the amino acids tryptophan is quite specific with respect to increasing the urinary excretion of nicotinic acid and N^1-methylnicotinamide.

One possible explanation for the favorable effects of cerelose in comparison to sucrose is that the cerelose contributed significant quantities of nicotinic acid to the diet. Microbiological analyses of the rations for two of the experimental series failed to demonstrate any greater amounts of nicotinic acid in cerelose than in sucrose. Further, it was recognized that the water of crystallization of cerelose (glucose monohydrate) increased the protein percentages of the rations slightly in that the water of crystallization was not taken into account in preparing these rations. This does not appear to be a major consideration, however, since animals fed 18 per cent casein with sucrose grew at a slower rate than animals receiving only 12 per cent casein with cerelose (Table I) and the values for the urinary excretion of nicotinic acid and N¹-methylnicotinamide were also in accord with the growth data (Table II). It seems likely, therefore, that cerelose favors the intestinal synthesis of nicotinic acid or its precursors to a far greater extent than sucrose. The apparent effect, therefore, of the kind of carbohydrate on the microbial synthesis of nicotinic acid or related substances is at least as great as that observed for riboflavin and thiamine (16–18).

Further evidence on the effect of the level and kind of protein on the growth rate and the urinary metabolites of nicotinic acid was obtained. The effect of feeding tryptophan was determined on the growth rate and urinary excretion of nicotinic acid and N¹-methylnicotinamide when a diet containing 12 per cent casein plus 12 per cent gelatin was fed for a period of 38 days (Table III). Animals that were fed tryptophan immediately resumed a rapid rate of growth and also excreted large amounts of nicotinic acid derivatives. It was demonstrated, therefore, that even when animals were fed a diet of this type a prompt and large increase in the excretion of these compounds occurred when tryptophan was added to the diet. The apparent conversion, therefore, of tryptophan to nicotinic acid was not impaired. An extension of the work with this and other diets showed that a response to feeding tryptophan occurred when varying levels of casein were fed (Table IV). The increase in the amounts of N¹-methylnicotinamide and nicotinic acid excreted occurred more slowly, however.

TABLE III

Effect of Tryptophan Ingestion on Growth and Nicotinic Acid Metabolism of Rats Fed Diets Containing 12 Per Cent Casein and 12 Per Cent Gelatin without Nicotinic Acid

Regimen	Days on experiment	Gain per day	Nicotinic acid excreted per day	N¹-Methylnicotinamide excreted per day
		gm.	γ	γ
Basal	14–17	1.5	11.0	24.7
"	23–26	0.5	7.0	24.6
"	35–38	0.3	6.7	21.3
" + tryptophan*	35–38	4.0	33.7	375
"	42–44	0.8	10.0	22.9
" + tryptophan*	42–44	3.5	179	2590

* 100 mg. of DL-tryptophan per rat per day. This sequence of treatment was carried out with one series of rats fed the basal diet for a previous period of 35 days. Comparable rats in the same series were continued as controls.

when 12 per cent casein and 12 per cent gelatin were fed than with the use of casein diets alone (see also Table III). The urinary values for nicotinic acid and N¹-methylnicotinamide appeared to be higher when the tryptophan was fed as the free amino acid than when an equivalent amount was fed as casein (Tables II to IV). This observation suggests that other amino acids in the additional casein and in the gelatin may decrease the amount of nicotinic acid excreted as compared to the effect when tryptophan only was fed. This may occur either by increasing the amount of nicotinic acid required to metabolize the increased quantities of amino acids ingested or by reducing the intestinal synthesis of nicotinic acid, or both.

TABLE IV

*Urinary Excretion of Nicotinic Acid and N¹-Methylnicotinamide by Rats Receiving Various Diets before and after Feeding Tryptophan**

Regiment†	Nicotinic acid excreted per day		N¹-Methylnicotinamide excreted per day	
	Before	After	Before	After
	γ	γ	γ	γ
12% casein	4.4	61.0	11.5	1200
18% "	6.1	49.3	15.6	847
24% "	8.2	53.8	37.4	904
12% " + 12% gelatin	6.3	22.2	13.3	285
12% " + 12% "		27.8‡		763‡

* 100 mg. of DL-tryptophan were fed per rat per day. The values given for tryptophan feeding are an average for the first 3 days of feeding.

† No nicotinic acid added.

‡ These values are for the second 3 days that tryptophan was fed (see also Table III).

Earlier work (1–8) which demonstrated that feeding tryptophan markedly increased the urinary excretion of nicotinic acid and N¹-methylnicotinamide was largely done with diets adequate in protein. Results obtained on the amounts of apparent free tryptophan excreted in the urine (19) showed that a very small amount of that ingested was excreted. Also, the excretion of certain other amino acids (phenylalanine, arginine, histidine, and threonine) was the same for animals receiving the gelatin or casein diets (20). As in earlier work (21), no difference was noted in the amounts of tryptophan excreted when cerelose or sucrose was used as the carbohydrate.

Studies with Mice and Cotton-Rats—Weanling albino mice and cotton-rats (*Sigmondon hispidus hispidus*) were used to obtain some comparative information. Studies were conducted with sucrose diets only. Mice fed 12 per cent casein and 12 per cent gelatin without nicotinic acid grew at a rate of 1.1 gm. per week (3 week period) and animals receiving nicotinic acid grew at a rate of 2.6 gm. per week. Animals fed 24 per cent casein without nicotinic acid grew at a normal rate (3.6 gm. per week).

Preliminary studies with cotton-rats revealed that animals receiving 12 per cent casein without nicotinic acid grew at a rate of 1 gm. per week, and when nicotinic acid was fed the growth rate was 4 gm. per week. When 18 per cent casein was fed, animals grew at a rate of 3.6 gm., and 6.1 gm. per week when nicotinic acid was added. No difference was noted when nicotinic acid was added to diets containing 24 per cent casein. Thus, the dietary requirement of the cotton-rat for nicotinic acid reported previously (22) has been confirmed. 18 per cent casein was used in both studies. The cotton-rat and mouse, therefore, like the white rat, do not need additional nicotinic acid when 24 per cent casein is fed and can also utilize tryptophan as a precursor of nicotinic acid (4, 12).

DISCUSSION

From the literature available it appears definite that for many species tryptophan fed either as the free amino acid or as a protein serves as a precursor for the vitamin nicotinic acid. The very rapid increases noted in the amounts of nicotinic acid and N^1-methylnicotinamide excreted when tryptophan is fed and the rapid reduction in the amounts excreted when tryptophan is removed from the diet clearly indicate that the transformation of tryptophan to nicotinic acid occurs within the tissues of the animal. Just how this occurs is not clear and final proof for this conversion remains to be obtained. Vitamin B_6 has been shown to be necessary for this conversion, since when tryptophan was fed to animals deficient in vitamin B_6, no appreciable increase in the urinary excretion of nicotinic acid or N^1-methylnicotinamide occurred (12, 13). When vitamin B_6 is added to the diet of animals deficient in vitamin B_6, an increase in the excretion of the nicotinic acid compounds occurs with the ingestion of added tryptophan. In the present work when rats were fed low protein diets or diets containing gelatin with tryptophan added, large increases were noted in the quantity of nicotinic acid derivatives in the urine. Whether only vitamin B_6-deficient animals are unable to convert tryptophan to nicotinic acid remains to be determined.

The marked influence noted with cerelose as the carbohydrate in the diet as compared to sucrose and the results obtained with gelatin show that factors other than the amount of tryptophan and vitamin B_6 ingested are involved. The most plausible explanation is that cerelose favors the microbial synthesis of nicotinic acid, thereby sparing the limited supply of tryptophan in low protein rations for uses by the body other than as a source of nicotinic acid. Other factors not at present recognized as being involved in this interrelationship may be contributed by the cerelose and not by sucrose. At any rate, cerelose does not appear to contribute the "pellegragenic" factor (23, 24), postulated as being present in corn, to the diet. The addition of gelatin to the diet counteracts the beneficial effects noted with cerelose (Tables I and II). The influence of gelatin appears to be similar to that observed with corn, acid-hydrolyzed proteins deficient in tryptophan, and glycine. It may be quite possible that less dietary nicotinic acid is needed for optimum growth when cerelose is fed with gelatin than when sucrose is fed.

The various interrelationships that have been demonstrated suggest many opportunities for further work. More rigorous proof is needed for the exact reactions involved in the pathways of metabolism of tryptophan and nicotinic acid.

SUMMARY

1. The effect of feeding diets containing graded levels of casein and different carbohydrates without added nicotinic acid on the rate of growth and urinary excretion of nicotinic acid and N^1-methylnicotinamide has been studied.

2. Animals receiving cerelose diets with 12 or 18 per cent casein grew at a more rapid rate than animals receiving sucrose. The amounts of nicotinic acid and N^1-methylnicotinamide excreted were in accord with the growth data.

3. The ingestion of diets containing 24 per cent casein allowed maximum growth, since no increase occurred with either sucrose or cerelose as the carbohydrate when nicotinic acid was added to the diet.

4. The addition of 12 per cent gelatin and 12 per cent casein to diets containing sucrose or cerelose depressed the rate of growth in the rat, mouse, and cotton-rat. This depression could be counteracted by feeding either nicotinic acid or tryptophan.

5. When tryptophan was added to diets containing varying quantities of protein, a marked rise in the urinary excretion of N^1-methylnicotinamide and nicotinic acid occurred in all cases.

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A CHEMICAL METHOD OF ESTIMATION OF NICOTINIC ACID IN URINE IN THE PRESENCE OF SUGAR

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It has been reported that intravenous injection of nicotinic acid produces hypoglycemia in normal humans (1–4). A definite improvement in the carbohydrate tolerance of diabetic patients after administration of nicotinic acid has also been reported (4). It was therefore of interest to study the urinary excretion of nicotinic acid in diabetic patients.

In the chemical estimation of nicotinic acid in urine by König's reaction (5) the urine is first digested either with acid (6) or with alkali (7) in a water bath to convert derivatives of nicotinic acid excreted in the urine to nicotinic acid. The color of the urine after digestion interferes with the estimation of nicotinic acid and different workers tried to remove the color by different methods. While Melnick and Field (6) and Swaminathan (7) used charcoal to remove the interfering pigments, Perlzweig, Levy, and Sarett (8) and Dann and Handler (9) employed Lloyd's reagent. Wang and Kodicek (10), on the other hand, treated the digested urine first with isobutanol, which removed some of the colors, and the remaining pigments were subsequently oxidized with potassium permanganate. Recently Swaminathan used zinc hydroxide to remove the color (11). The urine of diabetic patients, however, after digestion with acid or alkali gave a highly colored extract. The interfering color could not be removed by any of the methods so far used (6–11). It seemed that most of the color produced after digestion was due to charring of the sugar present in the urine. In the present paper a method for the estimation of nicotinic acid in the urine containing sugar has been described.

EXPERIMENTAL

Removal of Sugar in Urine by Treatment with Permanganate—Fresh solutions of glucose of different strengths were acidified with concentrated hydrochloric acid and treated in a water bath with a 10 per cent solution of potassium permanganate until the solution gave no test for sugar when boiled with Benedict's reagent. It was observed that approximately 16 cc. of a 10 per cent solution of potassium permanganate were required to oxidize 1 gm. of glucose. Urine containing glucose also required the same amount of permanganate solution as the pure solution of glucose of similar strength. When the sugar in the urine was oxidized with permanganate in an alkaline medium, colloidal manganese oxide was formed which was difficult to remove by centrifugation, and when the urine containing the manganese oxide was digested in the water bath, the manganese oxide was found to destroy the nicotinic acid, and added nicotinic acid could not be recovered. Sugar in the urine was therefore oxidized by permanganate in an acid medium.

Removal of Manganese in Urine after Treatment with Permanganate—When the permanganate-treated urine was digested in an alkaline medium, the manganese hydroxide formed did not destroy the nicotinic acid, but it was found difficult to remove and wash the bulky precipitate of manganese hydroxide either before or after the digestion. It was therefore thought desirable to remove the extra manganese in the urine before digestion. The manganese present in the urine was removed as phosphate by treatment with disodium hydrogen phosphate and NaOH. Crystals of disodium hydrogen phosphate were added to the urine treated with permanganate and heated in a water bath until the crystals went into solution. 0.35 gm. of disodium hydrogen phosphate was necessary for 1 cc. of 10 per cent permanganate. The hot solution was treated with 40 per cent sodium hydroxide drop by drop until the precipitation of manganese phosphate was complete. The precipitate was removed by filtering through a Büchner funnel under suction and washed twice with 10 cc. portions of distilled water. The filtrate was then ready for digestion.

Digestion of Sugar- and Manganese-Free Urine Filtrate and Removal of Interfering Color—In order to convert nicotinamide, nicotinuric acid, and N^1-methylnicotinamide into nicotinic acid the sugar- and manganese-free urine filtrate was put into a 250 cc. beaker. The solution was made alkaline with 40 per cent sodium hydroxide so that the strength of the alkali was 4 per cent and was digested in a boiling water bath for 45 minutes. After digestion the solution was neutralized with concentrated hydrochloric acid and an extra 3 cc. of acid were then added. The acidified solution was heated in the boiling water bath and treated with 10 per cent permanganate drop by drop until the solution became light yellow in color. The solution was then adjusted to pH 7 with bromothymol blue as an indicator and was made up to a definite volume. Phosphate buffer of pH 7 was then added to the solution in the proportion of 6 cc. of buffer per 10 cc. of the solution. A white precipitate appeared and the solution became almost colorless. The precipitate was filtered off and an aliquot of the filtrate was used for the colorimetric estimation of nicotinic acid.

Colorimetric Estimation of Nicotinic Acid—Nicotinic acid in the filtrate was then estimated according to the method of Swaminathan (7), slightly modified. 16 cc. of the filtrate were put into a 25 cc. stoppered graduated cylinder and into a similar cylinder was put a dilute solution of nicotinic acid, 1 cc. containing 10 γ of nicotinic acid. 6 cc. of phosphate buffer of pH 7 were added and the volume was made up to 16 cc. To each of the cylinders was added 1 cc. of a 4 per cent alcoholic solution of aniline, followed by 8 cc. of a solution of cyanogen bromide prepared freshly by decolorizing saturated bromine solution with 10 per cent sodium cyanide. The contents of the cylinders were shaken, allowed to stand for 2 minutes, and the yellow color which developed was then compared in a visual colorimeter. Sometimes the color of the urine filtrate was so light that a blank correction was not necessary. In case there was any residual color, the urine filtrate was treated exactly as described above, without the addition of cyanogen bromide, and compared with a standard nicotinic acid solution treated with cyanogen bromide.

Table I

Recovery of Nicotinic Acid Added to 25 Cc. of Urine Containing 5 Per Cent Glucose

Urine sample No.	Nicotinic acid added	Nicotinic acid estimated	Nicotinic acid recovered
	γ	γ	*per cent*
1a	0	25.8	
1b	30	55.5	99.0
1c	40	64.0	95.5
2a	0	50.0	
2b	15	63.5	90.0
2c	25	73.0	92.0
2d	10	59.3	93.0
3a	0	50.0	
3b	20	69.6	98.0
3c	40	89.0	97.5

Estimation of Nicotinic Acid in Urine in Presence of Sugar—Sugar in the urine is quantitatively estimated with Benedict's reagent. To 25 cc. of urine in a 250 cc. beaker are added 8 cc. of concentrated hydrochloric acid. The beaker is heated in a water bath and is treated with 10 per cent potassium permanganate until all the sugar is oxidized. 1 gm. of sugar requires approximately 16 cc. of a 10 per cent solution of potassium permanganate. The manganese present in the solution is removed as phosphate by treatment with disodium hydrogen phosphate and NaOH. The solution is then digested in a water bath for 45 minutes with 40 per cent NaOH so that the strength of the alkali is 4 per cent. The interfering color is removed by making the solution acidic with concentrated hydrochloric acid and treating with permanganate solution until the solution becomes almost colorless.

If the volume of the resulting solution is large, it is concentrated in a boiling water bath. The solution is adjusted to pH 7, phosphate buffer is added (6 cc. of buffer per 10 cc. of the solution), and the mixture filtered. An aliquot of the filtrate, depending on the amount of nicotinic acid present, is taken for the colorimetric estimation of nicotinic acid.

Recovery of Nicotinic Acid Added in Urine Samples—Nicotinic acid in different amounts was added to 25 cc. samples of urine containing 5 per cent glucose. Estimation of nicotinic acid in the samples of urine was carried out to learn how far this procedure could recover the added nicotinic acid in the urine. 90 to 99 per cent of added nicotinic acid could be recovered by the above method. The results are shown in Table I.

24 Hours Urinary Excretion of Nicotinic Acid by Diabetic Patients—24 hour urines of eight diabetic patients were collected in bottles containing 20 cc. of a 50 per cent solution of sulfuric acid. Nicotinic acid in these samples was estimated according to the method described above. Diabetic patients excreted 1.3 to 5.6 mg. of nicotinic acid per day. The results are given in Table II.

TABLE II

Excretion of Nicotinic Acid in Urine by Diabetic Patients (Males)

Patient	Age	Nicotinic acid excretion	Patient	Age	Nicotine acid excretion
	yrs.	*mg. per day*		*yrs.*	*mg. per day*
S. K. M.	60	2.8	J. C. D.	48	4.5
S. B.	30	4.8	R. K. G.	54	1.3
S. N. B.	47	2.6	R. B. S.	40	3.6
J. N. S.	51	5.2	M. N. M.	45	5.6

DISCUSSION

By preliminary treatment of urine samples containing sugar with permanganate, not only the sugar but also some of the interfering colors were removed. When urine of normal persons or rabbits was treated with 0.5 cc. of 10 per cent potassium permanganate in an acid medium and digested with alkali after removal of the manganese as phosphate, a lightly colored digest was obtained. This digest when treated with permanganate in acid medium became almost colorless. The method of estimation of nicotinic acid described in the present paper seems to be simplest of the existing chemical methods.

The daily urinary excretion of nicotinic acid by diabetic patients was found to vary between 1.3 and 5.6 mg. It was shown by one of us (N. C. G.) that normal healthy individuals excreted 1.4 to 5.3 mg. of nicotinic acid in their daily output of urine (12). Wang and Kodicek (10) reported that normal healthy British subjects excreted 0.85 to 2.33 mg. of nicotinic acid per day. Nicotinic acid nutrition of diabetic patients, therefore, does not seem to be interfered with.

SUMMARY

1. A chemical method of estimation of nicotinic acid in urine in the presence of sugar has been described.

2. The urine is first treated with permanganate, which removes sugar and some of the interfering colors. The manganese is removed as phosphate and the sugar-free urine is digested with alkali. The digested urine is decolorized with permanganate and the pH of the solution is brought to 7. After addition of phosphate buffer an aliquot of the solution is treated with alcoholic aniline and a solution of cyanogen bromide. The resultant yellow color is compared with a standard nicotinic acid solution similarly treated.

3. The urinary excretion of nicotinic acid by eight diabetic patients has been studied. The diabetic patients have been found to excrete 1.3 to 5.6 mg. of nicotinic acid per day. These figures compare well with the urinary excretion of nicotinic acid by healthy individuals.

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ISOLATION AND CHARACTERIZATION OF TAMARIND SEED (TAMARINDUS INDICA L.) POLYSACCHARIDE

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The alcohol-insoluble fraction from the water extract of tamarind seed meal, constituting 60 to 65 per cent of the husked kernel, has been described as a rich source of pectin (1–3). Although it forms a firm jelly in the presence of appropriate amounts of sugar and acid (1, 4) and has been suggested for commercial use as a substitute for pectin (5), it has been shown in preliminary communications by the authors (4, 6–8) and later by others (2, 3, 9–11) that it differs fundamentally from fruit pectins. Pectins are characterized by the presence of methyl ester groups and galacturonic acid units (12) with varying amounts of arabinose and galactose, presumably derived from associated araban and galactan, loosely attached to the molecule (13–15). On decomposition pectins also yield pectic acid, a product of definite chemical composition (16, 17), which is obtained either directly (18) or, better, through the insoluble calcium salt (19, 20) after hydrolysis by mild alkali followed by neutralization with acid. This paper records the results of investigations on the nature of the polysaccharide fraction of tamarind seed which show how in all these respects it differs from pectins, a conclusion necessitating revision of the views held hitherto to account for many of the phenomena of pectin-sugar-acid jelly formation (21, 22).

EXPERIMENTAL

The results of analysis of decorticated tamarind seed meal are given in Table I. The testas, which formed 28.5 to 33 per cent of the whole seed, were removed from the kernel after parching with sand, light crushing, and winnowing. All determinations were carried out according to methods of the Association of Official Agricultural Chemists (23); the values for the polysaccharide were obtained by precipitation of the aqueous extract with 50 per cent alcohol according to the method of Ghose and Krishna (1).

The alcohol precipitate yielded 8.57, 8.71, and 8.82 per cent respectively of crude proteins, expressed in terms of the weight of the original seed meal; these accounted for nearly half the total proteins of the seed meal and therefore also for the surplus over 100 per cent in Table I.

In view of the close protein-polysaccharide association (Table I), a fractionation study of the chief types of proteins in the seed meal appeared worth while. This was effected as follows:

20 gm. of the seed meal (Sample I) were extracted by refluxing with 80 per cent ethanol. The residue from the extract after removal of alcohol gave, on treatment with acidulated water or dilute alkali, a product with 0.22 gm. of total protein having certain of the characteristic properties of prolamins, which yielded on hydrolysis glutamic acid, proline, and ammonia.

The residual seed meal after extraction with alcohol was taken up in the cold in 2 liters of distilled water with a mechanical stirrer. A considerable part (8.1 gm.) of the polysaccharide and all of the albumins of the seed meal, amounting to 1.79 gm., were thus brought into solution; the latter could also be extracted with 5 per cent sodium chloride or 0.5 per cent sodium hydroxide and was completely precipitated on full saturation with ammonium sulfate.

Table I

Analysis of Tamarind Seed Meal

Determinations	Sample I	Sample II	Sample III
	per cent	*per cent*	*per cent*
Moisture	12.64	10.80	11.30
Ash, water-insoluble	3.73	4.37	3.87
" water-soluble	5.36	6.18	5.13
" total	8.09	10.55	9.00
" acid-insoluble	1.47	2.10	1.29
Polysaccharide (alcohol ppt., ash-free)	58.47	62.88	63.30
Crude proteins	15.69	15.28	15.35
Ether extractives	8.19	6.50	6.81
Crude fiber	4.93	1.46	2.60
Sugars	None	None	None
Tannins	"	"	0.83
Total	108.01	107.47	109.19

Ash present as Na, K, Ca, Mg, P, and Si.

The hot water-insoluble portion of the seed meal contained 1.13 gm. of glutelins insoluble in water, salt solution, and dilute alcohol, but soluble in dilute alkali.

Thus the proteins of tamarind seed meal consist of 1.1 per cent prolamin, 8.5 per cent albumin, and 5.65 per cent glutelin. It should, however, be stated here that these data were obtained by the application of conventional methods, no study having been given to the development of specific methods suitable for the extraction of the proteins of the tamarind seed.

Extraction of Gel-Forming Constituent—A convenient quantity of the seed meal (about 200 gm.) was made into a thin paste with the required quantity of cold water and poured slowly into about 5 liters of boiling water, the heating being continued for about 20 minutes. The mixture was then filtered and the solution, after treatment with sulfur dioxide to decolorize it, was concentrated to a small bulk *in vacuo* and the gel-forming constituent precipitated by addition of an equal volume of alcohol. The product, when dried, has the following per cent composition: moisture 2.90, ash 3.98, albuminoids 14.83, crude fiber 0.96, ether extractives 1.79; the rest constituted the polysaccharide fraction.

Preparation of Pure Polysaccharide—For the purification from its associated proteins of the fraction obtained as above, several methods were tried with varying degrees of success. The proteins were not removed, or even appreciably diminished on repeated solution in water and reprecipitation with alcohol or by fractional precipitation with alcohol of different strengths. Mild acid hydrolysis resulted in simultaneous degradation, to varying extents, of both the protein and polysaccharide constituents. The latter were also precipitated together from solution by treatment with protein precipitants such as phosphotungstic and tannic acids or by full saturation with ammonium and sodium sulfates. Prolonged digestion with proteolytic enzymes like pepsin and papain removed only 30 to 35 per cent of the nitrogenous fraction. Allowing the extract to stand overnight or using centrifugation for coagulation of the proteins was also only partially successful.

The most effective method of removal of proteins was found to be by considerable dilution of the aqueous extract. Thus, when the seed meal extract was diluted with increasing amounts of water and let stand overnight, increasing amounts of albuminoids settled; the supernatant liquid could be concentrated *in vacuo* after centrifuging and the gel-forming constituent precipitated with alcohol. Table II gives a typical set of results.

Dilution of the extract beyond 1:6 did not yield a purer product and, in various trials with different lots of seed meal, the purest product, obtained as above, yielded protein varying from 1.8 to 1.9 per cent. The jellies obtained with this preparation, in the presence of appropriate amounts of sugar and acid, had the maximum gel strength.[1]

Differences between Tamarind Seed Polysaccharide and Fruit Pectins—In spite of its undoubted property of forming sugar-acid jellies, the polysaccharide of tamarind seed differs fundamentally from fruit pectins, as may be seen from Table III; the purified pectins used for this study were prepared according to Savur and Sreenivasan (24).

In addition to the foregoing, the polysaccharide, unlike pectins, forms copper, calcium, and barium salts which separate as flocculent precipitates in alkaline medium. It also forms a highly viscous, gelatinous product when treated with small amounts of borax, resembling in this respect gum tragacanth. Pectic enzymes, such as pectin esterase and pectinase, which hydrolyze pectins to various cleavage products such as arabinose, galactose, and pectic or galacturonic acid (30–33), do not hydrolyze tamarind seed polysaccharide. On the other hand, taka-diastase, although without action on fruit pectins, hydrolyzes the polysaccharide with the liberation of a maximum of 86 per cent of reducing sugars. The purified product does not reduce Fehling's solution except when hydrolyzed with acids, whereas fruit pectins, even when highly purified, readily reduce Fehling's solution, evidently on account of the associated occurrence of free reducing sugars.

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[1] Savur, G. R., and Sreenivasan, A., unpublished work.

TABLE II

Variations in Protein Content with Dilution

1% seed meal extract, times diluted	0	2	3	4	5	6
Proteins in alcohol ppt., %	10.24	8.66	3.15	2.49	2.15	1.85

TABLE III

Comparison of Tamarind Seed Polysaccharide with Fruit Pectins

Determinations*		Apple pectin	Lemon pectin	Wood-apple pectin	Orange pectin	Tamarind seed polysaccharide
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Fresh basis	Moisture	11.6	9.3	4.1	8.7	4.0
Dry basis	Ash	2.22	1.05	2.98	2.38	3.05
	Ether extractives	0.83	1.87	0.90	2.13	1.60
	Alcohol ppt. (ash-free)	91.76	94.14	96.37	95.32	97.50
	Albuminoids	0.66	1.16	2.76	1.87	1.85
	Crude fiber	0	0	0	0	0
	Reducing sugars	12.50	20.72	22.40	26.82	0
	Calcium pectate (19)	80.35	91.72	91.96	89.60	0
	Pectic acid (16–18)	70.82	84.94	81.47	80.16	0
	Galacturonic acid (25)	24.20	26.49	22.48	23.21	0
	Mucic acid (26)	24.70	28.81	28.08	29.22	18.84
	Uronic acid (27)	41.80	62.20	58.70	62.90	3.44
	Pentosans (28)	12.14	15.47	18.28	16.97	27.54
	Methyl ester groups (29)	7.10	9.38	8.76	9.07	0

* *Cf.* (23). The figures below in parentheses refer to bibliographic references.

It would appear therefore that classification of tamarind seed polysaccharide as a pectin would rest solely on its property of forming sugar-acid jellies (2, 3); such a classification, based purely on physical behavior and not on chemical constitution, would be haphazard and confusing (34), especially since the Committee on Pectin Nomenclature of the American Chemical Society has clearly defined the group designation for pectins (35).

The polysaccharide cannot be classed as a mucilage either (10), for recent work has shown that substances commonly described as gums and mucilages are in fact polyuronides and that their acidic properties are due to the presence of uronic acid; hence the name, "acidic polyuronides" sometimes given to them ((34) p. 122). In an earlier communication (6), the authors had referred to the polysaccharide as a gluco-galacto-xylan and there is little doubt that it is a "polyose" or "hexopentosan" (34), a nomenclature which is based on hydrolytic and oxidation studies referred to below.

Products of Hydrolysis—As already stated, tamarind seed polyose does not reduce Fehling's solution unless hydrolyzed by dilute acids. Reducing sugars formed on hydrolysis by boiling, under a reflux, a 1 per cent suspension for different periods of time with 2 and 4 per cent sulfuric acid are given in Table IV. The hydrolysates were partially neutralized with baryta and, towards the end, with barium carbonate, filtered, and the sugars in the filtrate determined by Bertrand's method (36). For comparison, the results of hydrolysis of fruit pectins with 4 per cent acid are included.

TABLE IV

Acid Hydrolysis of Tamarind Seed Polyose and of Fruit Pectins

Time	Sugars, expressed as glucose						
	Tamarind seed polyose		Apple pectin	Wood-apple pectin	Lemon pectin	Orange pectin	Glucose and xylose
	Sulfuric acid, per cent						
	2	4	4	4	4	4	5
min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	0	0	12.5	14.4	20.2	11.8	29.9
60	12.3	17.3	16.6	16.2	23.8	14.1	28.4
120	22.5	28.4	18.8	19.2	26.1	16.8	27.7
180	34.9	41.3	22.4	21.5	27.2	19.9	25.2
240	35.5	37.4	25.9	24.4	29.1	22.9	24.4
300	34.9	37.1	25.9	26.8	30.1	27.7	20.7
480	25.6	32.7	37.4	32.7	37.4	31.0	17.1

It is evident that hydrolysis with acids does not result in quantitative formation of reducing sugars and that prolonged hydrolysis results in partial destruction of sugars, an observation reported earlier (37) and confirmed by the results of hydrolysis, for varying periods, of a mixture of equal parts of glucose and xylose with 5 per cent sulfuric acid (included in the last column of Table IV). In a number of trials, the maximum amount of reducing sugars found in hydrolysates of tamarind seed polyose was about 41 per cent at the end of 6 hours boiling with 3 or 4 per cent sulfuric acid.

Using 1 per cent extract of tamarind seed meal and hydrolyzing with 0.1 gm. of taka-diastase at 37.5° for 24 and 48 hours gave 74.7 and 86.7 per cent, respectively, of reducing sugars.

Identification of Sugars—The only sugars that could be identified in the acid or enzymic hydrolysate, obtained as above, were glucose, galactose, and xylose. The hydrolysate was neutralized, filtered, and concentrated *in vacuo*. An aliquot of this concentrate was heated on a water bath and treated with phenylhydrazine and glacial acetic acid. The mixture was allowed to react for 45 minutes and then cooled. The osazones of the hexoses that crystallized were removed in batches and recrystallized. Glucosazone (m.p. 204°) and galactosazone (m.p. 155.5°) were the only products identified, although the latter was, to a certain extent, contaminated with xylosazone.

Another aliquot of the concentrated hydrolysate was fermented with yeast to remove the hexoses and at the end of 24 hours the solution was treated as above for the osazone reaction. Only xylosazone (m.p. 154°) could be obtained in this way. The presence of xylose was confirmed by Bertrand's reaction (38) with the help of bromine and cadmium carbonate, when characteristic boat-shaped crystals, identified under the microscope, of the double salt cadmium bromide-cadmium xylonate were obtained. The absence of arabinose was conclusively proved by the fact that no characteristic diphenylosazone could be obtained (39). It is possible, although not probable in the light of the evidence presented here, that there may be other components of the polyose which have escaped detection.

The data for reducing sugars in the acid hydrolysate were obviously unsuited for the quantitative characterization of the polyose, in view of the destruction, to varying degrees, of the sugars under these conditions (Table IV). Calculations based on specific rotation of acid or enzymic hydrolysates were also ruled out, as it frequently happens that small experimental errors are enormously magnified and that the final results, even with mixtures of pure sugars, can be regarded as only roughly approximate (36). The actual quantities of galactose and glucose in the polyose were therefore ascertained from a study of the yield of mucic and saccharic acids on oxidation with nitric acid, while that of xylose was deduced from the yield of furfural on distillation with dilute hydrochloric acid. The procedures are detailed below.

Products of Oxidation—5 gm. of the seed polyose were treated with 300 cc. of 25 per cent nitric acid (sp. gr. 1.15) and the mixture was heated on a water bath for about 3 hours, when its volume was reduced to 50 to 75 cc. On dilution, mucic acid separated out ((36) p. 691). This was allowed to stand for 2 hours and then filtered. The precipitate was dissolved in dilute sodium carbonate solution, filtered, and the mucic acid reprecipitated by addition of a slight excess of hydrochloric acid. It was then filtered, dried, and weighed. The product had a melting point of 225–227°, corresponding to mucic acid. It was quantitatively determined according to Tollens (26).

The filtrate obtained after removal of mucic acid was neutralized by the addition of potassium carbonate, treated with a few drops of acetic acid, and evaporated to a syrupy consistency. After addition of a few more drops of acetic acid, it was then allowed to cool, when potassium acid saccharate separated out. This was filtered and recrystallized from a small amount of water. The acid salt was neutralized with ammonia and the excess ammonia removed by boiling. On addition of silver nitrate, a white precipitate was obtained. This was separated, dried, and carefully ignited. The residue was cooled and weighed. The equivalent weight of the acid was calculated from the formula

TABLE V

Products of Oxidation

Substance	Mucic acid	Saccharic acid
	per cent	*per cent*
Apple pectin	24.70	0
Wood-apple pectin	28.08	0
Orange pectin	29.22	0
Lemon "	28.81	0
Tamarind seed polyose	18.84	64.59

$$\text{Equivalent weight} = \frac{\text{Weight of Ag salt}}{\text{Weight of Ag}} - 1$$

A value of 206.56, corresponding to that of saccharic acid (210), was obtained.

The yields of mucic and saccharic acids, obtained as above, are given in Table V in comparison with the values for mucic acid of purified fruit pectins (24); the latter did not yield saccharic acid.

Probable Molecular Proportion of Sugars in Polyose—The purified polyose on distillation with 12 per cent hydrochloric acid yielded furfuraldehyde, which as phloroglucide amounted to 30.0 per cent (26). The xylose equivalent of this was calculated from Krober tables ((36) p. 1276). From this and from the yields of mucic and saccharic acids (Table V) obtained on oxidation with nitric acid ((36) p. 691) the amounts of xylose, galactose, and glucose were calculated to be 28.42, 16.19, and 55.36 per cent respectively;

these values correspond approximately to a molecular ratio of 2:1:3 o xylose-galactose-glucose. On the basis of the results recorded here th polyose may therefore be classed as a xylo-galacto-glucosan. There is n evidence on record of a similar polyose with these sugars as component ((34) p. 70). The constitution of the polyose remains to be worked out but, considering the fibrous nature of the product and its gel-forming property, it may be presumed to have a chain structure rather than a ring structure (40).

SUMMARY

1. An aqueous extract of tamarind seed meal gives, on precipitation with 50 per cent alcohol, 60 to 65 per cent of a polysaccharide which forms jellies with sugar and acid as in the case of fruit pectins.

2. The polysaccharide obtained as above is associated with 14 to 15 per cent of crude proteins, accounting for nearly half of the total seed proteins: the jelling property is not, however, related to the protein fraction.

3. The polysaccharide could be freed from most of the proteins by excessive dilution (1:100 or above), followed by standing overnight to coagulate the latter.

4. The purified polysaccharide is free from methyl ester groups and galacturonic acid units, does not give the Carre and Haynes reaction for pectins, and differs also in other respects from fruit pectins.

5. The only sugars formed on acid or enzymic hydrolysis of the polysaccharide are xylose, galactose, and glucose. From a quantitative study of the products of oxidation and hydrolysis, it is concluded that the approximate molecular proportion in which these sugars are present in the polysaccharide is 2:1:3 respectively.

6. The polysaccharide is designated as a "polyose" or "hexo-pentosan" which may be specifically termed gluco-galacto-xylan.

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ELECTRON MICROSCOPE STUDIES OF SODIUM HYALURONATE

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PLATE 1

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Considerable interest is attached to the acid polysaccharides because of their presence in the ground substance of connective tissue, synovial fluid, cartilage, vitreous humor, cornea, umbilical cord, certain bacteria, and in many tumors (1, 2). They also may possibly be involved in the pathology of the rheumatic and "pararheumatic" diseases (3–5). Hyaluronic acid and hyaluronidase also play an important rôle in fertilization (6) and are probably involved in fluid transfer in the tissues (7, 8).

Information is available concerning some of the physical properties of hyaluronic acid. From the high viscosity, streaming birefringence, and ability to spin into threads it may be concluded that the extracted hyaluronate has a fibrous structure. From viscosity and streaming birefringence data, Blix and Snellman (9) estimate a mean particle length for the purified neutral hyaluronate of 4700 A with a maximum above 10,000 A. These solutions are polydisperse. The variation in particle length and uniformity depended on the source of the material and the method of preparation. Assuming unbranched chains and a disaccharide length of 10 A, they estimate the particle weight to range between 200,000 and 500,000. The degree of polymerization is also greatly affected by pH, ionic environment, oxidizing agents, and specific depolymerases (9–11).

Since electron microscopy is capable of revealing structure in the colloidal range, it seemed desirable to investigate the properties of hyaluronic acid with this technique. The results here reported serve chiefly as an orientation for further detailed studies which must be undertaken if the full potentialities of the method are to be brought to bear on the problem.

Methods and Materials

Three specimens of hyaluronate prepared by different techniques in different laboratories were examined. The electron micrographs shown here were taken of a highly purified, protein-free preparation from umbilical cord, the chemical composition of which corresponds closely to the theoret-

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* This work has been done during the tenure of a Life Insurance Medical Research Fund Fellowship.

ical values. The method of preparation of this sample is as yet unpublished.[1] A drop of approximately 1:10,000 sodium hyaluronate in twice distilled water was allowed to dry on the supporting film of the specimen grid. In order to increase contrast and show contour the preparation was then shadowed by the method of Williams and Wyckoff (12) with 8 mg. of chromium at an angle of 9° and at a filament to object distance of 18 cm. An RCA type EMB electron microscope was used in this study.

Results

All three samples showed the same general characteristics. The polysaccharide dries inhomogeneously on the film. In some regions of higher concentration (not shown) it forms thin, apparently structureless sheets of uniform thickness and density. At lower concentrations long, branching, and anastomosing fibers predominate (Fig. 1). In some areas stellate sheets interconnected by fibrous processes are observed (Fig. 2). Frequently globular enlargements occur along the course of a fiber, suggesting relatively higher fluidity in these regions (Fig. 3). The individual fibers usually have a smooth contour and are fairly constant in width over their extent. Observed widths range from approximately 50 to 200 A. No regular lateral or axial structure was observed in this material.

The structure of the supporting collodion film is responsible for the fine, pebbly background as indicated by electron micrographs of shadowed blanks. Other small, dense, irregular and unidentified bodies are probably contaminants. The small rod-shaped structures in the background of Fig. 2 were noted in this picture only and their significance is unknown.

DISCUSSION

The particular patterns observed in the electron micrographs are probably determined to a certain extent by the method of preparation (drying from an aqueous solution). The fibrous structures probably result from flow processes produced by interfacial tension during drying. It is thought that the thin, elongate filaments which give rise to viscosity and streaming birefringence in solution align themselves to form larger aggregates when subjected to relatively small shearing forces. Whether or not aggregates of this particular structure exist naturally or in solution is undetermined.

The presence of globular enlargements is reminiscent of similar structures observed in the electron micrographs of rubber (13). If a similar interpretation is applied, one may suppose that the membranous, globular, and fibrous patterns represent fractions of polysaccharide differing in degree of polymerization or particle length. The fibrous processes would consist of a parallel array of the longest particles. The globules would be composed of short units; hence they show more fluid properties.

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[1] The author is deeply indebted to Dr. Karl Meyer of the College of Physicians and Surgeons, Columbia University, for the material from which these pictures were obtained. He also wishes to thank Dr. Walter Bauer of the Massachusetts General Hospital and Dr. Maxwell Schubert of the Department of Therapeutics, New York University College of Medicine, for other samples of purified polysaccharide used in **this study.**

The fibrous processes do not show the discrete, filamentous characteristics of proteins such as actin, myosin (14), and insulin (15).[2] Hyaluronate fibers anastomose and branch rather than cross discretely over each other as do these filamentous proteins.

Investigations of the macromolecular structure of other acid polysaccharides are in progress as well as the effects of pH, oxidizing agents, specific enzymes, and combinations with protein.

SUMMARY

1. Electron micrographs of purified sodium hyaluronate were obtained from aqueous solutions dried on the supporting film and shadowed with chromium.

2. Characteristic structures include thin, anastomosing, fibrous processes, globular enlargements, and stellate sheets.

3. These structures are interpreted to represent processes incident to preparation, which cause the polysaccharide chains of various lengths to take these forms. These are probably related to the degree and form of polymerization.

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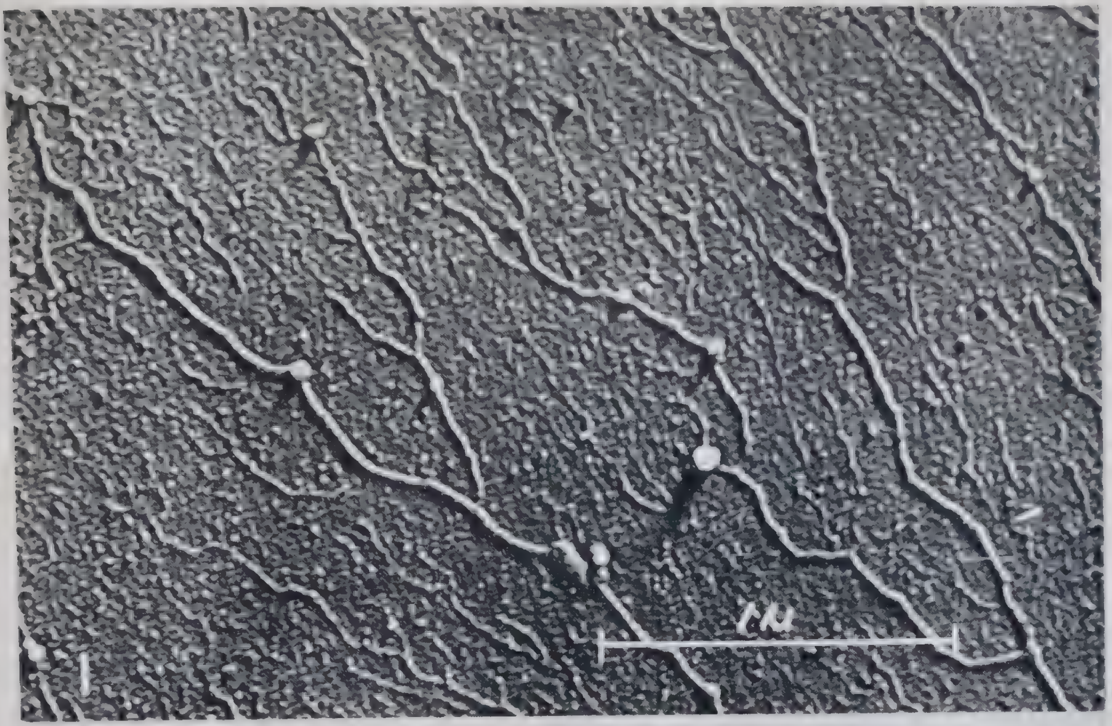
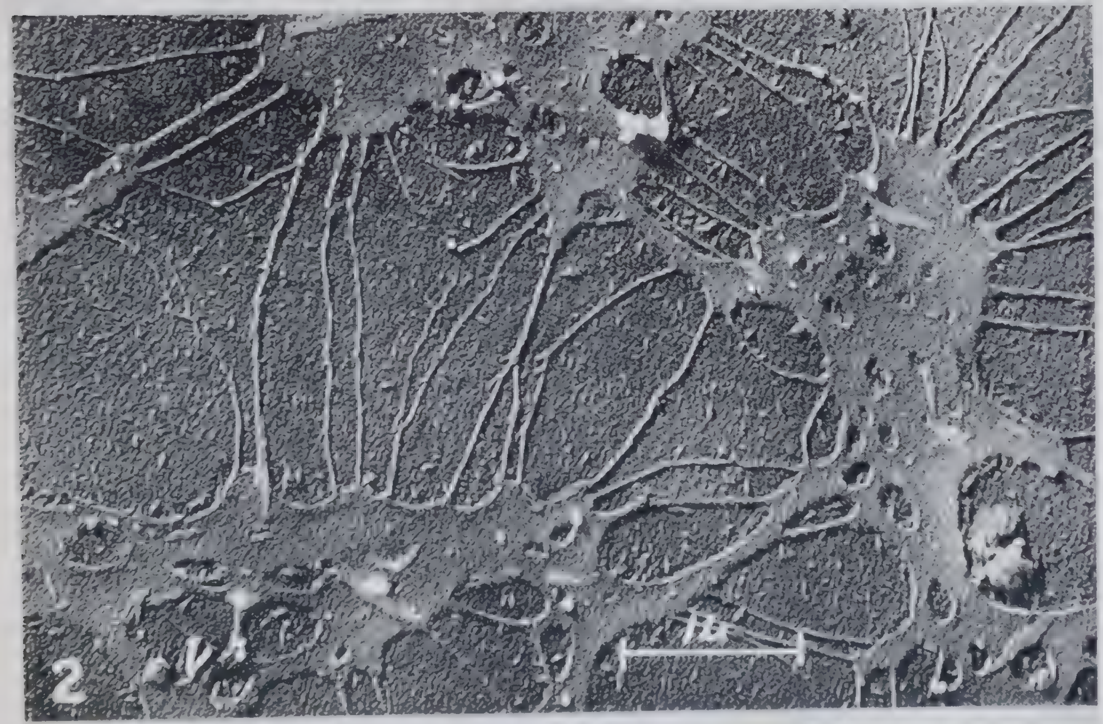
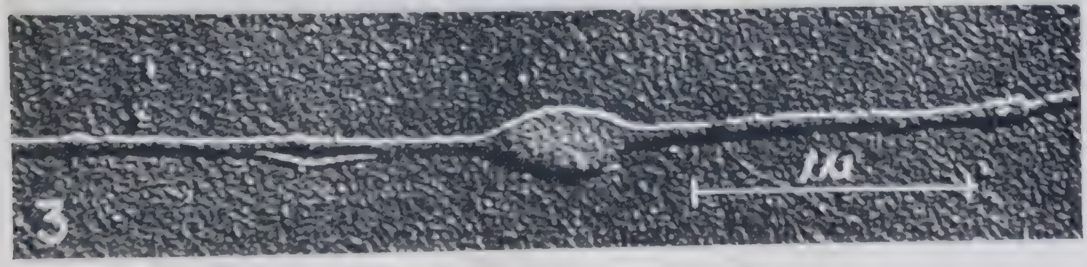
EXPLANATION OF PLATE 1

Figs. 1 to 3. Sodium hyaluronate from umbilical cord 1:10,000 (approximately) in water, dried on film; three different regions of the same specimen grid. Shadowed with chromium. Magnification, 48,000 (Fig. 1), 24,000 (Fig. 2), 38,000 (Fig. 3).







(Gross: Electron microscope studies of sodium hyaluronate)

EFFECTS OF FEEDING INDIVIDUAL AMINO ACIDS UPON THE DISTRIBUTION OF OTHER AMINO ACIDS BETWEEN CELLS AND EXTRACELLULAR FLUID

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Amino nitrogen is present in much higher concentration in tissues than in plasma (1–3). Nevertheless injected amino acids are taken up by the cells so that the tissue concentrations are increased more than the plasma concentration (1). Glutamine is present at higher concentrations in the tissues than in the plasma of dogs (3). In contrast the sum of the concentrations of valine and leucine is not appreciably higher in the tissues than in the plasma of guinea pigs (4).

Although the tissue amino acids appear to be free by customary analytical methods, they may be present in some unstable combination which does not pass through the cell wall. Thus, administered creatine enters cells against apparent concentration gradients to form phosphocreatine (5, 6). Such unstable compounds of the amino acids might be identical with the high energy intermediates postulated in peptide bond formation (7), for example N-phosphoryl compounds. We have been interested in the possibility that the magnitude of the distribution ratios of amino acids between cells and extracellular fluids might determine whether growth or wasting occurs.

In the present communication evidence is reported that glycine is distributed according to a characteristic ratio between the cells (liver and muscle) and the extracellular fluid. Amino acids which produced high concentrations in the organism when fed produced a diminution of the distribution ratio (cells to extracellular fluid) for glycine. Similarly when high glycine levels were produced by feeding glycine, the distribution ratios for the non-glycine amino acids were reduced. Of the amino acids tested, only glutamic acid produced *increases* rather than decreases in the distribution ratios of the three categories of amino acids determined. We interpret these findings to mean that each of the amino acids, when at high concentration, competes with other amino acids for the means employed by cells for the concentration of amino acids, and that L-glutamic acid in some way contributes to the intracellular concentration of other amino acids.

EXPERIMENTAL

Guinea pigs of both sexes (300 to 550 gm., Rockland Farms) were maintained upon the Rockland guinea pig diet with greens in addition two or three times a week. Amino acids and sodium benzoate were administered by mouth to animals after a 12 hour fast, in either a neutral solution or a suspension by a dropper pipette, and washed down with water. Feedings were practically quantitative. Dosages were usually 10 to 20 millimoles of amino acid per kilo of body weight. With a view to having more or less steady absorption from the gut for nearly 3 hours, two-thirds of the total amount of amino acid was given at the beginning of the experiment, one-sixth an hour later, and one-sixth 2 hours later. The animals were sacrificed at 3 hours. Omitting the later two portions and sacrificing the animals at 2 hours did not modify greatly the effects observed with glycine.

Other experimental details have been described (8), including the determination of glycine (9) and of glutamine α-amino nitrogen and non-glutamine α-amino nitrogen, both at pH 4.7 with ninhydrin (10). The α-amino nitrogen was thus divided into three categories, that due to glutamine, that due to glycine, and that due to neither, which for convenience will be called the "residual" α-amino acid nitrogen. Glycine was recovered as well (101 ± 3 per cent) from the picric acid-lead acetate filtrates at various levels of enrichment of tissues, as from standard solutions. Corrections (small, but appreciable) were made routinely for the formaldehyde obtained in the absence of ninhydrin. The specificity of the glycine method (9, 11) is supported by the observation that the deduction of the values for glycine nitrogen from the values for the total α-amino nitrogen eliminated most of the variability of the latter values. To enhance the precision of the glycine determination, aliquots usually were selected which gave optical transmittances between 45 and 60 per cent.

The concentration of each category of nitrogen in mg. per gm. of cell water was calculated from the proportions of extracellular and intracellular water, determined according to Lowry and Hastings (12, 8). The water distribution was determined in the tissues of the control animals and of those receiving sodium benzoate or glycine, but average normal values were assumed for other animals when it became apparent that variations in water distribution were not of significant magnitude. The differences reported have been checked for statistical significance (13).

Peptides in Tissues—We have attempted to measure peptides in tissues by analyzing filtrates prepared with picric acid, followed by neutral lead acetate (10). The picric acid filtrates contained conjugated α-amino nitrogen not passing through cellophane equivalent to about 2 mg. per 100 gm. of tissue, indicating about 99.9 per cent removal of protein. The lead acetate treatment, however, left only 0.02 mg. per cent of non-dialyzable conjugated α-amino nitrogen, so that dialysis of these filtrates was unnecessary.

The following results for conjugated α-amino nitrogen were obtained: five guinea pigs, liver 0.2 mg. per cent ($\sigma = 0.2$), muscle 3.2 mg. per cent ($\sigma = 1.1$), kidney 1.0 mg. per cent ($\sigma = 0.8$); mouse carcass 1.9 mg. per cent, mouse carcass with skin 7.0 mg. per cent.

The lead acetate precipitation, designed to remove the abundant glutathione, undoubtedly removed other peptides. From a fibrin hydrolysate, for example, containing about two-thirds of the amino acids in conjugated form, 42 per cent of the conjugated amino acid nitrogen was removed by lead acetate under conditions simulating those used in preparing tissue filtrates. One may conclude that tissues, especially muscle, contain appreciable concentrations of non-protein amino acid conjugates other than glutathione. Anfinsen and Beloff[1] have concurrently made similar observations upon the livers of rats.

Effect of Lead Acetate Treatment upon Free Amino Acids—The lead acetate filtrates were not ideal for the measurement of free amino acids but seemed preferable to glutathione-rich filtrates. About one-tenth of the α-amino acid nitrogen was lost from plasma by the treatment, and 14 per cent from a fibrin hydrolysate. The amino acids removed most extensively by lead acetate when they were added to plasma and tissue filtrates were glutamic acid (60 to 80 per cent), aspartic acid (50 to 70 per cent), and histidine (about 50 per cent), as indicated by the loss of α-amino nitrogen. Glycine, alanine, serine, phenylalanine, and proline were not lost to a measurable extent; methionine, tyrosine, and leucine suffered losses of 10 per cent or less. For many of the plasma samples, α-amino nitrogen was determined both before and after lead acetate treatment; the only plasmas showing severe losses were those obtained after histidine feeding.

RESULTS AND DISCUSSION

The glycine nitrogen of the plasma of normal guinea pigs varied for unknown reasons from 0.4 to 2.6 mg. per cent; the coefficients of variation (σ/mean) were 0.60, 0.50, and 0.52 respectively for glycine in plasma, liver cells, and muscle cells. Glycine contributed most of the variability of the amino acid concentrations of these tissues (Table I). The variations in glycine, however, *occurred together in the three tissues*, so that coefficients of correlation among them were above 0.9, or, expressed in another way, the coefficients of variation of the distribution ratios were 0.17 and 0.25.

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[1] Personal communication.

The liver cell tended to contain 33 times as much glycine as the extracellular fluid; the muscle cell, 8.3 times as much. The residual amino acids showed distribution ratios of 15.7 (σ = 3.0) and 5.3 (σ = 1.1) for liver and muscle respectively. The residual amino acids were not sufficiently variable to establish that their changing values in the liver, muscle, and plasma were positively correlated. The surprisingly large proportion of the free amino acids represented by glycine (31 to 63 per cent of the non-glutamine α-amino nitrogen in liver, 19 to 56 per cent in muscle) may be related to the relatively large contribution of glycine to urinary amino acid conjugates in man (11).

TABLE I

Effects of Ingestion of Benzoate (300 Mg. per Kilo) and of Glycine, Glutamate, and Aspartate (Each 20 mM per Kilo) upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

The mean values are given, followed by the standard deviations.

	Glutamine	Glycine		"Residual"	
	α-Amino N in cell water	α-Amino N in cell water	(Cellular)/(Extracellular)	α-Amino N in cell water	(Cellular)/(Extracellular)
	mg. per cent	*mg. per cent*		*mg. per cent*	
Controls (13)*					
Liver	5.5 ± 1.4	32 ± 16	33 ± 5	38 ± 6	15.7 ± 3.0
Muscle	5.5 ± 1.8	8.6 ± 4.5	8.3 ± 2.0	13.2 ± 2.9	5.3 ± 1.1
Benzoate (3)†					
Liver		9.0 ± 4.2	19 ± 2.4	51 ± 12	25 ± 7
Muscle		7.6 ± 3.0	16.5 ± 1.6	27 ± 14	13.5 ± 8
Glycine (4)					
Liver		129 ± 13	5.5 ± 0.7	22 ± 9	7.0 ± 2.6
Muscle		41 ± 8	1.7 ± 0.2	9.0 ± 1.8	3.0 ± 0.4
Glutamate (5)					
Liver	10.3 ± 2.1	33 ± 6.2	40 ± 3.8	49 ± 4.4	21 ± 3.7
Muscle	8.5 ± 2.4	10.0 ± 2.2	12.2 ± 2.0	21.5 ± 5.5	7.9 ± 1.1
Aspartate (3)					
Liver		37 ± 3	31 ± 2	44 ± 1.4	21 ± 3
Muscle		10.6 ± 1.7	8.8 ± 0.9	17 ± 1.8	7.9 ± 1.5

* The figures in parentheses represent the number of animals.

† Animals sacrificed 70, 80, and 120 minutes after feeding sodium benzoate.

Sodium Benzoate—A dose of sodium benzoate (300 mg. per kilo of body weight) which should stimulate a nearly maximum rate of synthesis of hippuric acid reduced the free glycine of liver and secondarily that of plasma, so that the distribution ratios, liver cell to plasma, and muscle cell to plasma, moved downward and upward respectively (Table I). This illustrated what happens when the withdrawal of an amino acid is stimulated in one of the two tissues, and is to be contrasted with the effects of L-proline or DL-serine, for example, which caused both liver and muscle cells to lose glycine (see below). The results indicate a predominance of the liver over the kidneys (14) in hippuric acid synthesis in the guinea pig, the distribution ratio for glycine between kidney and plasma being little changed. Accumulation of hippuric acid in the liver (measured as conjugated amino acid nitrogen extractable by ethyl acetate) was small, in one case 1.7 mg. per cent of nitrogen.

L-*Amino Acids*—Of the amino acids administered (excepting DL forms) L-proline, L-histidine, glycine, and L-methionine caused large rises in plasma amino acids. These were also the amino acids giving the largest rises in the amino acids of liver and muscle cells. As the glycine concentration of the plasma was increased after glycine feeding, the distribution ratios for glycine were decreased (although the absolute increase in the glycine concentration was always greater in the cells than in the plasma). Similarly, at high concentrations of residual α-amino nitrogen, the distribution ratio of this category was reduced. This would be expected if the capacity of cells for concentrating each of the amino acids is limited. But whenever large elevations of the plasma glycine were produced, the distribution ratios of the other (residual) amino acids, between the cells (liver and muscle) and the plasma, were also depressed (Table I), with an accompanying rise in plasma concentration. Similarly, whenever the plasma residual amino acids were elevated, the distribution ratio of *glycine* between liver

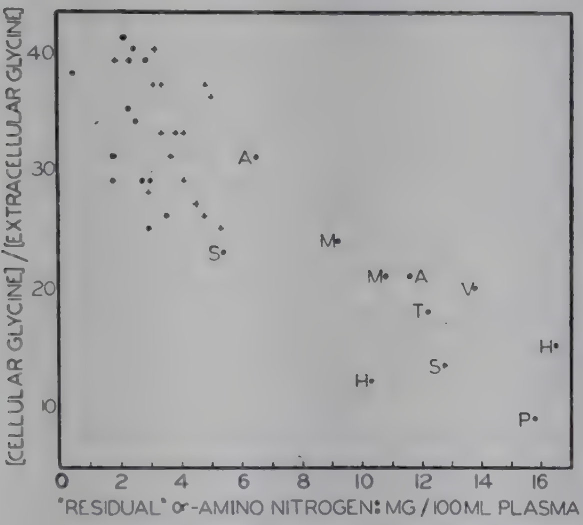

FIG. 1. Relation of the distribution ratio for glycine between liver cells and extracellular fluid, to the plasma residual α-amino acid nitrogen after the feeding of individual amino acids. A = DL-alanine; H = L-histidine hydrochloride; M = L-methionine; P = L-proline; S = DL-serine; T = DL-threonine; V = DL-valine. +, experiments with other amino acids; ●, fasting controls.

cells and extracellular fluid was decreased approximately in proportion (Fig. 1). The ingestion of L-proline caused the plasma glycine nitrogen to rise to the extraordinary level of 7.7 mg. per cent with the distribution ratios falling below one-third of normal. An effect upon the muscle glycine was not apparent under the conditions of the experiments, except in the case of proline.

All the other L-amino acids examined gave undetectable to moderate increases in the plasma α-amino acid nitrogen, smaller effects upon the α-amino acid nitrogen of liver cells, and no significant effect upon the distribution of glycine. The majority of the amino acids tested caused increases of the α-amino nitrogen of muscle cells of the guinea pig (*cf.* (2)). Several of the differences in plasma response to ingested amino acids recorded here have been noted before in other species (15–23).

DL-*Amino Acids*—In cases where comparisons were made (alanine, serine, leucine, and phenylalanine), the DL-amino acids elevated the plasma amino acid concentration more than the corresponding L acids. The intense effect upon glycine distribution produced by high accumulations of an amino acid was also obtained with DL-amino acids (threonine, serine, valine, and alanine), muscle as well as the liver being affected (Table II; Fig. 1).

The amino acids appear to show competitive inhibition among themselves for the means by which the cells concentrate amino acids, indicating that these means do not operate independently for each amino acid. An identical mechanism could scarcely concentrate each of the large number of amino acids and yet maintain characteristic distributions for each in the face of the variations of the amino acid mixtures presented. Thus, a high plasma glycine level reduced the distribution ratio of other amino acids much less than that of glycine itself. The slope indicated roughly by the points of Fig. 1 suggests that an increase of about 9 mg. per cent of α-amino nitrogen due to a single amino acid in the plasma (*i.e.*, about 9 times the glycine concentration) halves the ability of the liver cell to hold glycine. Protein synthesis obviously requires the presence in some form and at some limiting concentration of every amino acid (essential or non-essential) which must be built into the molecule. As a corollary, it may be inferred that, when a strongly asymmetric accumulation of amino acids is produced by feeding or injection, the ability of the cells to retain other amino acids will be handicapped and one may expect an inhibitory effect upon growth or nitrogen retention. Thus, the difficulty in producing optimum growth of rats with diets containing gelatin supplemented with amino acids, or with diets containing 5 to 6 per cent of glycine, L-proline, or DL-phenylalanine (24, 25), may well be an effect of the excessive accumulation of one or more amino acids. The amino acids producing high blood concentrations upon feeding are the ones which cause extra energy production ("heat of specific dynamic action") and in some cases extra urea excretion (16, 26).

Table II

Effects of Ingestion of Several Other Amino Acids upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

Because of the great variability in glycine concentrations the distribution ratio (glycine nitrogen per 100 gm. of cell water to 1.05 × mg. of glycine nitrogen per 100 ml. of plasma) appears more useful. For the residual α-amino acids the cellular concentration is the more useful value because of its relative constancy. Mean values followed by standard deviations are given where two or more animals are grouped.

Amino acid fed	Dosage, mM per kilo	Glycine			Residual α-amino N		
		Plasma	Liver	Muscle	Plasma	Liver	Muscle
		α-Amino N	Distribution ratio	Distribution ratio	α-Amino N	Concentration in cell water	Concentration in cell water
		mg. per cent			*mg. per cent*	*mg. per cent*	*mg. per cent*
Fasting (13 animals)		1.02	33	8.6	2.46	38	13.2
		±0.61	±5	±2.1	±0.53	±6	±2.9
L-Alanine	10	0.92	31	8.6	3.86	46	24
" *	10	0.73	26	7.1	4.76	34	22
DL-Alanine*	10	1.22	31	6.2	6.5	46	21
" *	20	1.52	21	6.4	11.6	41	31
L-Serine	12.5	1.95	28	6.5	2.92	60	20
DL-Serine*	12.5	0.82	37	8.0	3.19	62	19
"	12.5	2.4	23	5.9	5.39	64	22
DL-Serine*	20	3.1	13.5	3.4	12.8	95	21
DL-Threonine*	15	1.89	18	8.0	12.2	93	23
DL-Valine*	15	1.0	20	6.6	13.8	49	28
L-Leucine (3 animals)	10	1.24	33	9.9	4.4	34	15.2
		±0.46	±6	±1.7	±0.9	±2	±4.7
DL-Leucine*	10	0.51	39	11	6.54	35	21
L-Proline*	15	7.7	9	2.2	15.8	72	30
L-Methionine (2 animals)	10	0.85	22	9.8	10.0	83	23
		±0.05	±2	±1.0	±0.8	±2	±1
L-Phenylalanine*	10	0.81	33	8.6	3.81	42	17.0
DL-Phenylalanine*	10	0.99	36	9.7	5.00	43	17.9
L-Tyrosine	10	0.95	29	10.3	4.1	38	19.2
L-Tryptophan	10	0.77	37	11	4.8	38	18.5
L-Ornithine	6.4	0.76	37	10.9	3.12	48	20.6
L-Lysine	10	1.96	27	9.2	4.29	43	16
L-Arginine (2 animals)	10	0.94	33	10.1	2.6	48	17
		±0.15	±1	±0.3	±0.8	±7	±4
L-Histidine (2 animals)	10	1.07	13.7	9.7	13.4	76	24
		±0.12	±1.5	±0.5	±3.1	±11	±1

* Basal ration supplemented with pyridoxine, 5 mg. per kilo.

The elevation of the alanine concentration of plasma when glycine is ingested by man, and of the glycine concentration when DL-alanine is fed (13), is an additional illustration of the response of cells to high concentrations of an amino acid. Decreases in the plasma concentration of several amino acids in dogs after DL-methionine feeding were noted by Hier and Bergeim (21). Humans fed glycine showed increased amino acid conjugates in plasma ((11); *cf.* (17)). The conjugated amino acids of an ultrafiltrate of the plasma of a dog were doubled after a dose of L-glutamic acid which elevated the portal α-amino nitrogen to 10.7 mg. per cent.[2] High plasma concentrations of an amino acid may provoke loss of peptides as well as of free amino acids from cells.

The observations recorded here may also assist in explaining the urinary losses of amino acids in mice fed peroxide-treated proteins (27), and the production of vomiting during the infusion of DL-amino acids and of L-glutamic and L-aspartic acids (28–30).

L-*Glutamic Acid*—The failure of L-glutamic acid absorption to increase noticeably the amino nitrogen of peripheral blood was noted by Seth and Luck (16). Increases occurred in portal blood, however. In our experiments, none of the three fractions of the amino acids of cardiac plasma was increased during glutamic acid absorption by guinea pigs; in fact, the residual amino acids were decreased (means, fasting, 2.46 mg. per cent; after glutamic acid, 2.01 mg. per cent).

In a partially gastrectomized dog receiving by mouth 20 mM of L-glutamic acid (as sodium glutamate) per kilo of body weight, we observed, in collaboration with Dr. Charles Dent,[2] a net decrease in the glycine, glutamine, and residual α-amino nitrogen in both the portal and jugular plasma, following preliminary increases, especially of the residual α-amino nitrogen in the portal plasma.

The explanation, we believe, lies in an increased uptake of various amino acids by tissues when glutamate is fed. Each of the three categories of amino acids determined was increased in liver and muscle cells of guinea pigs, and each of the distribution ratios was elevated while glutamate was being absorbed. Glycine does not appear to undergo transamination with the glutamate-α-ketoglutarate system (31, 32).

After feeding L-aspartic acid, as with L-glutamic acid, the plasma concentrations were not elevated, whereas the residual amino acids in liver and muscle were increased. The distribution of glycine was unaffected, however.

It is concluded that glutamate (or α-ketoglutarate) is somehow involved in the system by which the cells concentrate the amino acids presented to them in the extracellular fluid. Whether the observed effects are related to the conversion of glutamate to α-ketoglutarate (transamination) or to the disposition of the α-ketoglutarate with resultant formation of high energy phosphate compounds, or to other reactions, remains to be determined.

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[2] Unpublished results with Dr. Charles Dent.

SUMMARY

1. Glycine represented a large and highly variable portion of the amino acids of skeletal muscle and liver of guinea pigs. The glycine of the muscle cells, liver cells, and plasma were covariant, so that fairly constant distribution ratios were maintained. The glutamine levels of these tissues were also highly variable but with little covariance among them. The concentrations of the non-glutamine, non-glycine ("residual") amino acids were moderately constant.

2. Those amino acids which upon feeding caused high plasma concentrations (L-proline, L-histidine, glycine, L-methionine, DL-threonine, DL-serine, DL-valine and DL-alanine) caused the largest increases in the amino acids of liver and muscle cells.

3. When high glycine levels were produced by feeding glycine, not only was the distribution of glycine affected but the distribution ratios of the residual amino acids (cellular to extracellular) were also reduced.

4. Those amino acids which produced high concentrations in the organism caused a reduction not only of the distribution ratios for the category to which they belong but of the glycine distribution ratios as well. Those amino acids which gave small rises in the plasma concentration had little if any effect upon glycine distribution.

5. Glutamic acid alone of the amino acids studied produced an *increase* in the distribution ratios of each of the three categories of amino acids determined. The plasma amino acids were *diminished* during glutamate absorption.

6. These observations are interpreted to mean that the various L- and D-amino acids compete with each other, although only to a restricted extent, for the means by which cells concentrate the amino acids presented to them by the extracellular fluid, and that L-glutamic acid contributes in some way to this concentrating mechanism.

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THE ACCUMULATION OF OROTIC ACID BY A PYRIMIDINELESS MUTANT OF NEUROSPORA*

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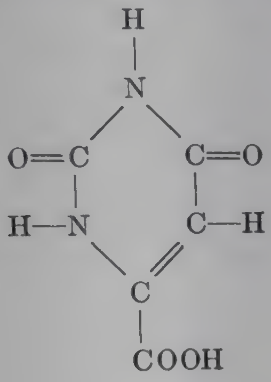
The discovery of orotic acid (I, 4-carboxyuracil) in cow's milk by Biscaro and Belloni (1), followed by its identification and synthesis (2–4), led to a number of speculations as to its biological origin and significance (3, 5, 6).



(I)

A definite connection of orotic acid with the biosynthesis of nucleic acid pyrimidines is provided by the finding that orotic acid (7) as well as thymine (8, 9) can supplement or replace the folic acid required by certain microorganisms. As suggested by Chattaway (7), it would appear that folic acid has a function in the biosynthesis of pyrimidines. Furthermore, this function is probably concerned in some step prior to the appearance of orotic acid in the biosynthetic series.

More recently it was shown by Loring and Pierce (10) that orotic acid could be substituted for uracil in satisfying the growth requirements of some *pyrimidineless* mutants of the mold *Neurospora*.

Investigations on orotic acid in this laboratory have led to a new method of synthesis of the compound (11) and to some suggestions concerning its relation to the biosynthesis of nucleic acids in *Neurospora* (12). The results of the present work are in accord with the previous suggestions and provide further evidence on the biological origin and function of orotic acid.

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EXPERIMENTAL

Accumulation of Orotic Acid—Orotic acid has been found to be accumulated in large quantities by three mutants of *Neurospora*. Strain 38502 has been previously described with respect to genetic constitution (13) and growth characteristics (12). Strains 36601 and 37709 have not been considered before, but they may be tentatively assumed to be an independent recurrence of the same mutation as that in strain 38502. During growth in the presence of cytidine these mutants excrete orotic acid into the culture medium in which it can be determined by means of its absorption spectrum (14) or by its growth-promoting effect on mutant 263 (12). The maximum quantity produced in experiments thus far carried out is 1.3 mg. per ml. of culture fluid. This quantity is slightly less than the maximum solubility of orotic acid at 25°.

A variety of culture conditions and methods of isolation has been investigated. In most cases mutant 38502 was grown at 25° from a conidial inoculation of 15 liters of minimal medium (15) supplemented with 250 mg. of cytidine sulfate. After 4 to 7 days growth under forced aeration the mycelium was filtered off and discarded, since it contained only a small amount of orotic acid. Nearly all of the compound is accumulated during the rapid growth of the first 4 days. A better yield was obtained when the culture fluid contained 40 gm. of $CaCO_3$. This substance serves to maintain the pH of the medium above 5.5 and thus allows a more luxuriant growth of the mold. Orotic acid is also produced in large quantities when strain 38502 is grown in the presence of corn steep liquor, yeast extract, or liver extract.

Isolation—Although several methods of isolation have been utilized, the simplest consists of crystallization of the sparingly soluble potassium salt from the culture fluid after evaporation to a relatively small volume. As an example, 7.5 liters of culture fluid, containing 6.9 gm. of orotic acid as determined by absorption spectrum analysis, were evaporated in a laboratory vacuum flash evaporator to 800 ml. The salt began to crystallize before the evaporation was completed. After cooling, the potassium orotate was filtered and air-dried. The yield of crude product (better than 90 per cent pure) was 6.3 gm., calculated as orotic acid.

Orotic acid may also be isolated by adsorption on charcoal, followed by elution with aniline or ammonia. These procedures have no apparent advantage over the direct method when one is dealing with relatively small volumes of medium.

Orotic acid may be obtained from any of its salts by boiling a short time in 300 parts of 2 N HCl. On cooling, the acid crystallizes with 1 molecule of water that requires temperatures above 110° for removal.

Identification of Isolated Compound—The substance isolated was recrystallized twice from water and dried at 135° under a vacuum. The product gave the following analyses: C 38.41, H 2.75, N 17.70; calculated for orotic acid, C 38.48, H 2.56, N 17.94. Absorption spectra in 0.1 M HCl and 0.1 M NaOH were identical with those previously reported (11, 14) for synthetic orotic acid. The isolated material was treated with Br_2 according to the procedure of Wheeler (2), giving a compound with the following analyses: N 9.84, Br 55.97; calculated for 5,5′-dibromobarbituric acid, N 9.78, Br 56.00. M.p. 236–239°; a mixed melting point with 5,5′-dibromobarbituric acid gave no depression.

TABLE I

Production of Orotic Acid by Pyrimidineless Mutants and Double Mutants

Strain	Temperature of growth	Orotic acid	Orotic acid*
	°C.	*γ per ml.*	*γ per ml.*
38502	25	610	
38502-1048-5†	25	1100	
38502, 263	25	0	
38502, 37301	25	0	
38502, 67602	35	0	20
38502, 37815	35	0	80
263	25	0	
37301	25	0	
67602	35	0	0
37815	35	0	0

* After growth at 35° cultures of these mutants were placed at 25° for 2 days.

† A reisolated strain of the original strain 38502.

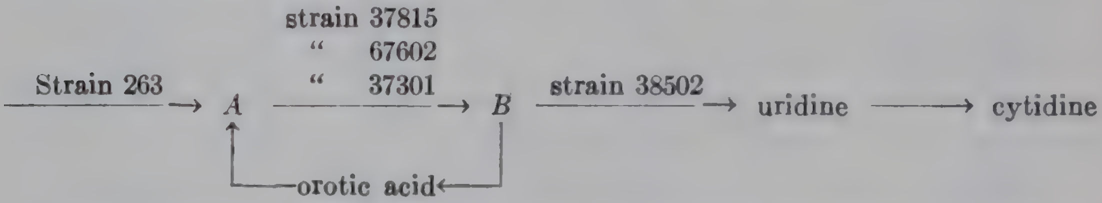
When tested for biological activity on *Neurospora* mutant 263 (12), the isolated compound induced the same amount of growth as a sample of synthetic orotic acid.

Orotic Acid in Uridine Biosyntheses—In a previous publication (16) use was made of double mutants of *Neurospora* for establishing the order of reactions controlled by several different genes concerned with a series of reactions leading to the biosynthesis of adenine. In a similar fashion it would be expected that mutant genes concerned with reactions in uridine biosynthesis, coming before that controlled by strain 38502 (orotic acid producer), would block the formation of orotic acid in the double mutant with strain 38502. Mutant genes concerned with reactions following those giving rise to orotic acid should not affect the formation of the compound by a double mutant. Preparation and genetic analyses of double mutants of strain 38502 with strains 263, 37301, 67602, and 37815 have been described elsewhere (13). Production of orotic acid by these mutants is summarized in Table I. The cultures were grown in 20 ml. of medium containing 0.4 mg. of cytidine sulfate. It should be noted that strains 67602 and 37815 are alleles of strain 37301, but they carry a partial block and no genetic block, respectively, in uridine synthesis at 25°.

From these data and those previously presented it is evident that biosynthetic reactions controlled by the mutations represented by strains 263 and 37301 come before that of strain 38502 in a series.

DISCUSSION

Although early investigations had provided an indication that orotic acid has a significant biological importance, the only direct evidence of its occurrence in biological systems was its isolation from milk (1). The present work, which demonstrates the production of large quantities of this pyrimidine by some mutants of *Neurospora*, establishes beyond question that orotic acid does have a significant place in the biosynthesis of nucleic acids. The evidence from work on the *Neurospora* mutants suggests the following scheme in the origin and utilization of orotic acid:



It appears probable that orotic acid is not actually an intermediate in the series of reactions, since it is not utilized by strain 37301, though it is accumulated by strain 38502. The reaction controlled by the wild type allele of strain 37301 must come before that of strain 38502, since the double mutant does not produce orotic acid. From this conclusion it is necessary that the reaction from *B* to orotic acid is irreversible. The evidence that strain 263 comes before strain 37301 in the series rests on the fact that strain 263 is able to utilize orotic acid for growth.

It is possible that the orotic acid in cow's milk arises from a side reaction in the biosynthesis of nucleic acid in the animal, analogous to the accumulation of the compound by some of the *Neurospora* mutants.

SUMMARY

1. It has been shown that three of the *Neurospora* mutants that require uridine, cytidine, or uracil for growth accumulate large quantities of orotic acid during growth. The acid is found almost entirely in the culture fluid.
2. Isolation and identification of the substance are described.
3. The relationship of orotic acid to nucleic acid biosynthesis is discussed.

It appears probable that orotic acid is a by-product and not a normal intermediate in the biosyntheses.

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BIOTIN AND THE METABOLISM OF LACTOBACILLUS ARABINOSUS*

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It is known that the requirement of microorganisms for certain growth factors is influenced by the nutritive medium in a manner analogous to the effect of dietary regimen of animals on their vitamin requirements. In this connection it was found by Williams and Fieger (1) that oleic acid could substitute for biotin in the growth of *Lactobacillus casei* under their experimental conditions. Axelrod *et al.* (2) have recently shown that this is also the case for *Lactobacillus arabinosus*. Koser *et al.* (3) were able to demonstrate that, for *Torula cremoris*, aspartic acid could largely substitute for biotin. More recently Stokes, Larsen, and Gunness (4) have shown a definite relationship between biotin and aspartic acid and were able to demonstrate that aspartic acid synthesis resulted when biotin was present. Since Williams and Fieger (1) demonstrated that oleic acid does not cause biotin synthesis, it appears possible that biotin is responsible for synthesis of both aspartic acid and oleic acid.

The purpose of the investigation herein reported is to elucidate the relationship between biotin and these two nutrilites and to determine whether there is a relationship between aspartic acid and oleic acid other than their common connection with biotin.

Methods

The cultures were handled in the manner usually employed in microbiological techniques (5). The agar stabs were of the following composition: glucose 1 per cent, sodium acetate 1 per cent, K_2HPO_4 0.5 per cent, Difco solubilized yeast extract 0.5 per cent, Bacto-tryptone 1 per cent, Salts B 1 per cent, vitamins equivalent to the high biotin medium and agar 1.5 per cent. The daily transfer medium from which the inoculum was prepared was the same as the basal medium, except that it contained 1.0 mg. of DL-aspartic acid and 2.0 mγ of biotin per 10 ml. tube. All growth experiments were carried out in Evelyn colorimeter tubes and readings were made at 660 mμ against a blank of uninoculated medium set at 100

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on the galvanometer. Each tube was inoculated with 1 drop of a 0.9 per cent saline suspension of a 12 to 24 hour culture of the organism. The density of the inoculum was adjusted to a galvanometer reading of 85 to 95 compared to a tube of water set at 100. The organisms were incubated at 37° for 60 hours, unless otherwise indicated.

The composition of the double strength basal medium is given in Table I. Biotin-free, low biotin, and high biotin media were prepared by adding 0, 0.5, and 20 $m\gamma$ of biotin respectively to each 10 ml. tube.

TABLE I

*Composition of Double Strength Medium**

	mg.		*mg.*
DL-Leucine	200	Glycine	100
DL-Isoleucine	200	Adenine sulfate dihydrate	20
DL-Valine	200	Guanine monohydrochloride dihydrate	20
L-Cystine	100	Uracil	20
L-Tyrosine	100	Xanthine	20
DL-Methionine	200		*ml.*
DL-Tryptophan	50	Salts A (6)	10
DL-Phenylalanine	200	" B (6)	10
	gm.		*gm.*
L-Glutamic acid	1.0	Glucose (anhydrous)	20
	mg.	Sodium acetate (anhydrous)	20
DL-Threonine	200		γ
L-Lysine monohydrochloride monohydrate	200	Calcium pantothenate	200
L-Arginine monohydrochloride	200	Riboflavin	200
L-Histidine monohydrochloride monohydrate	100	Thiamine hydrochloride	200
L-Proline	100	Nicotinic acid	200
DL-Serine	100	Pyridoxamine dihydrochloride	400
	gm.	*p*-Aminobenzoic acid	40
DL-Alanine	1.0	Folic acid	2.0
		Biotin as indicated	

* Adjust to pH 6.8 and make up to 500 ml.

In previous work (1, 2) the oleic acid-sparing effect was observed on a medium containing apparently adequate amounts of aspartic acid; therefore an attempt was made to discover whether oleic acid was effective on an aspartic acid-free medium. The results are shown in Table II. It may be noted that only poor growth resulted in the presence of aspartic acid or oleic acid separately on this biotin-free medium, but when both were present fairly good growth resulted. This growth was improved somewhat by adding Tween 80,[1] a non-ionic detergent which makes oleic acid more available to the organism (7). Under these conditions oleic acid and aspartic acid almost completely replace biotin. It can be seen in Table II that oleic acid and aspartic acid gave about the same growth on the biotin-free medium that aspartic acid gave on the low biotin medium. Since previous attempts to demonstrate biotin synthesis from oleic acid have failed (1), it is hardly surprising that oleic acid was ineffective in the absence of aspartic acid. Indeed this experiment provides additional evidence that oleic acid is not a precursor of biotin, for, if it were, aspartic acid would have been formed and growth would have ensued. This biotin-sparing action of oleic acid and aspartic acid can also be shown in another way. If an attempt is made to set up a biotin standard curve with and without aspartic acid, it is apparent that about 10 times as much biotin is required for maximum growth in the case in which aspartic acid is absent.

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[1] Atlas Powder Company product kindly supplied by Dr. E. E. Snell.

TABLE II

Replacement of Biotin by Oleic Acid and Aspartic Acid for Lactobacillus arabinosus

Supplement	Biotin level		
	None	Low	High
None	1.4*	1.9*	17.5*
Oleic acid, 0.4 mg.	1.0	4.2	18.3
DL-Aspartic acid, 2.0 mg.	4.6	14.4	19.0
Oleic + aspartic acid	12.8	13.0	19.7
" + " " + Tween 80, 3.0 mg.	15.7		

* Ml. of 0.1 N acid produced per 10 ml. tube in 4½ days.

This is shown in Fig. 1. In this experiment the amount of biotin required for half maximum acid production was 0.44 and 5.9 mγ in the presence and absence respectively of aspartic acid. In either case the maximum amount of 0.1 N acid produced was 20 ml. per 10 ml. tube. Results similar to these were obtained by Stokes *et al.* (8). From these experiments it appears that oleic acid replaces the small amount of biotin required in the presence of aspartic acid. Oleic acid and aspartic acid will nearly replace the larger amount of biotin corresponding to the biotin standard curve in the absence of aspartic acid. Since it has already been pointed out that biotin is involved in aspartic acid synthesis, a possible interpretation here would be that biotin is also involved in oleic acid synthesis. The small amount of biotin required when aspartic acid is present would correspond to the amount required for oleic acid synthesis. The larger amount of biotin required when aspartic acid is absent corresponds to the amount required for synthesis of both oleic acid and aspartic acid. It is apparent that under these experimental conditions the biotin requirement for activities other than aspartic acid synthesis is relatively small.

At this point the biotin-aspartic acid relationship was investigated. Stokes *et al.* (4) have shown that for several organisms aspartic acid synthesis occurs in the presence of biotin. Lyman *et al.* (9) have shown that for one of these, *Lactobacillus arabinosus*, both vitamin B_6 and carbon dioxide are required for aspartic acid synthesis. This suggested that biotin might be involved in carbon dioxide fixation in the synthesis of aspartic acid. Since a number of heterotrophic organisms are able to fix carbon dioxide by the β-carboxylation of pyruvic acid to form oxalacetic acid which can be transaminated to aspartic acid, it appeared that the primary reason these organisms failed to grow on a biotin- and aspartic acid-deficient medium was their inability to fix carbon dioxide. The effect of several substrates on the growth of the organism on low and high biotin medium was studied. The results shown in Table III indicate that oxalacetic acid can partially replace aspartic acid on the low biotin medium. The failure of the organisms to synthesize aspartic acid on the low biotin medium must be a result of their inability to form oxalacetic acid. It can be seen from Table III that bicarbonate, which had little or no effect on growth in the low biotin medium, had a pronounced effect on growth in the high biotin medium. Pyruvic acid had no effect on either medium apparently because it was present in sufficient concentrations at all times.

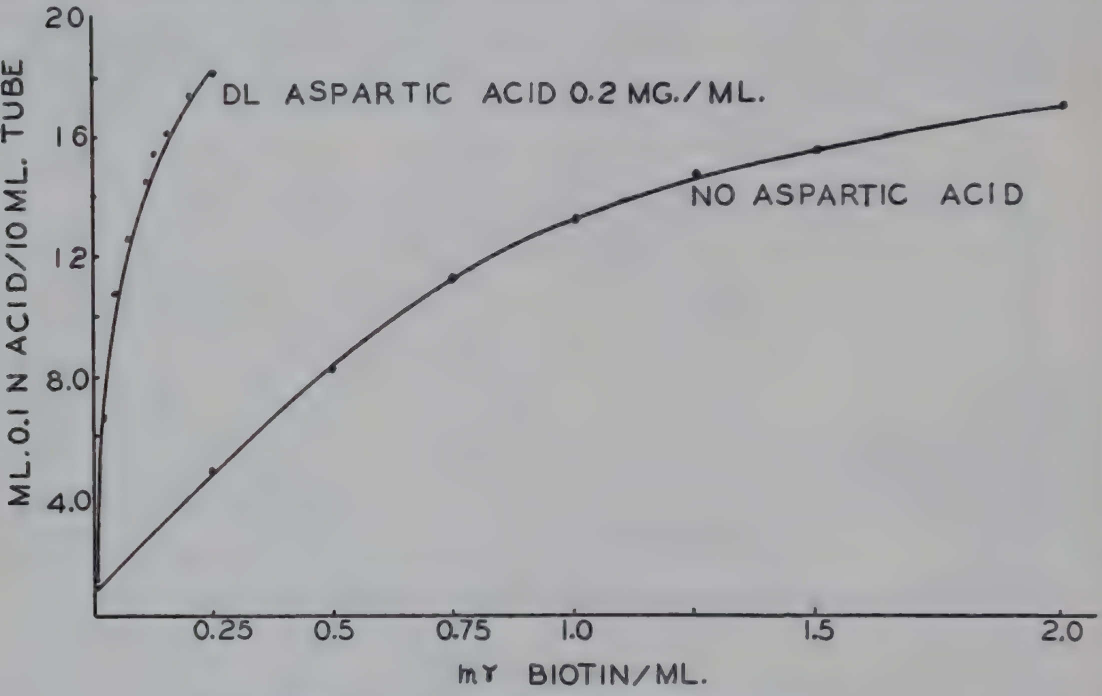

FIG. 1. Effect of aspartic acid on biotin requirement

The failure of oxalacetic acid to give as good growth as aspartic acid may be due to its lability. Furthermore, some organisms are known to contain an active oxalacetic decarboxylase (10). The best results were obtained by addition of small quantities of oxalacetate for the first 20 hours of the experiment. Additional amounts of pyridoxamine or pyridoxal did not increase growth in the presence of oxalacetic acid, which indicates that transamination was probably not the limiting reaction. Under these conditions β-alanine gave no stimulation, but rather appeared to be slightly inhibitory. Since the medium contained large amounts of α-alanine, the synthesis of aspartate by direct carboxylation of either of these compounds appears to be ruled out. The 4-carbon dicarboxylic acids, fumaric, malic, and succinic, were also inactive.

TABLE III

*Effect of Various Supplements on Low and High Biotin Media for Lactobacillus arabinosus**

Supplement	Low biotin medium		High biotin medium	
	10 hrs.	24 hrs.	10 hrs.	24 hrs.
None	94	90	93	44
$KHCO_3$, M/270	91	89	70	30
Oxalacetic acid, M/270	87	84	67	29
Aspartic acid, 2.0 mg	84	72	78	25
β-Alanine, M/500	94	91	93	59

* Galvanometer readings.

It appeared that these organisms were coupling carbon dioxide and pyruvate in the presence of biotin to synthesize aspartate. Therefore, an attempt was made to halt the synthesis on high biotin medium by reducing the carbon dioxide tension to a very low level. However, upon aeration with carbon dioxide-free air no growth took place in any tube. Apparently the organism would not grow under these conditions; so an attempt was made to reduce the carbon dioxide tension in small increments by progressively lowering the pH of the medium. The results are shown in Fig. 2. From pH 6.5 to 5.5 growth was nearly independent of acidity in the presence of the 4-carbon compounds, oxalacetic acid and aspartic acid. However, when the carbon chain of aspartic acid had to be synthesized, a sharp break occurred in the growth of the organism between pH 6.0 and 5.8. Whether this effect was due to reduced carbon dioxide tension or to the effect of low pH on the enzyme which forms oxalacetate (11) is not known.

Burk and Winzler (12) suggested earlier that biotin might function as a general carbon dioxide donor. A preliminary investigation was made of other biological syntheses involving the addition of 1 carbon atom. Of those reactions studied biotin did not appear to be involved in the conversion of anthranilic acid to indole or the formation of phenylalanine from β-phenylethylamine and carbon dioxide. The experimental organisms would not convert succinic semialdehyde[2] to α-ketoglutarate. For some inexplicable reason ornithine was not converted to citrulline and arginine could not be synthesized under the experimental conditions of Lyman (9), although citrulline readily replaced arginine.

DISCUSSION

The anomalous finding that either bicarbonate or oxalacetate gave better results for early growth than the compound they appear to be incorporated into was interpreted as being largely a carbon dioxide effect.

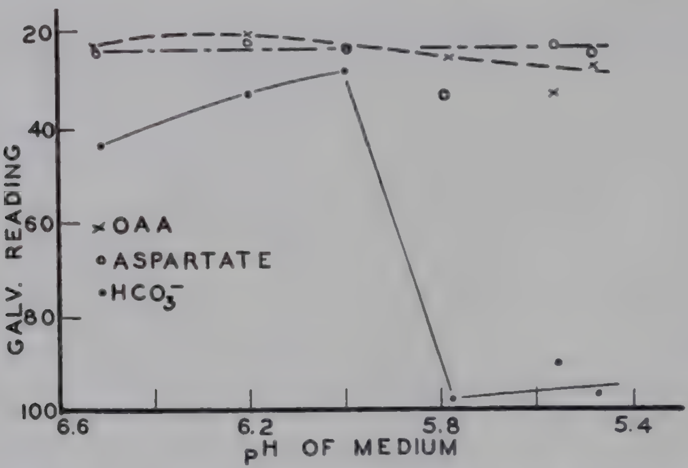

FIG. 2. Effect of pH on bicarbonate utilization on high biotin medium

Gladstone *et al.* (13) have shown that carbon dioxide often reduces the lag phase of bacterial growth. However, oxalacetate is often slightly more active than aspartate plus bicarbonate, which might possibly indicate that oxalacetate is used as a building block for compounds other than aspartic acid. Mitchell *et al.* (14) has suggested that oxalacetate may be a starting material for uracil synthesis in *Neurospora*.

While it may be too early to speculate on the interrelationships among biotin, aspartic acid, and oleic acid, several points stand out at this time. Although either oleic acid or aspartic acid spares biotin, they cannot replace each other. Since biotin appears to cause the synthesis of aspartic acid, this may also be the case with oleic acid or other fatty acids. Certainly oleic acid is not a precursor of biotin, as evidenced by the work of Williams and Fieger (1) and the data presented here. While no evidence has been presented to show that biotin causes oleic acid synthesis, the

[2] Generously furnished by Dr. Simon Black, University of Chicago.

biotin-oleic acid relationship appears to be analogous to the biotin-aspartic acid relationship. If biotin is involved in both aspartic acid and oleic acid syntheses, it appears that they do not arise by the same mechanism. Fig. 2 shows that conditions which alter aspartic acid synthesis do not affect the oleic acid-replacing activity of biotin.

On the basis of recent experiments Williams and Fieger (15) have suggested that biotin may not be a component of an enzyme system but rather is involved in cell permeability or surface action. However, until it is shown that the detergents in their experiments are active in the absence of oleic acid or other fatty acids nutritionally important to the organism, their data are compatible with a nutritional rôle for biotin and oleic acid. Williams, Broquist, and Snell (7) have shown that inactive detergents are active in the presence of oleic acid for some *Lactobacilli*. Certain other *Lactobacilli* have a nutritional requirement for oleic acid even in the presence of biotin (7, 16).

SUMMARY

1. Aspartic acid and oleic acid can almost completely replace biotin for the growth of *Lactobacillus arabinosus*.

2. The biotin requirement of *Lactobacillus arabinosus* for aspartic acid synthesis is at least 10 times as great as that for the other functions of biotin.

3. At pH 5.8 or below biotin can no longer cause aspartic acid synthesis, probably because of low carbon dioxide tension. The oleic acid-replacing activity of biotin is not affected by changes in pH in this range.

4. Biotin appears to catalyze the β-carboxylation of pyruvic acid.

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THE EFFECT OF PANTOTHENIC ACID DEFICIENCY ON ACETYLATION IN RATS*

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Lipmann *et al.* have shown (1) that coenzyme A[1] contains large amounts of pantothenic acid. Since this coenzyme has been shown to be essential for *in vitro* acetylations of sulfanilamide (3, 4) and choline (5), it seems significant to determine whether acetylation in animals depends upon an adequate intake of pantothenic acid.

Methods

The ability of normal and pantothenic acid-deficient rats to acetylate a foreign substance was compared by injection of *p*-aminobenzoic acid (PAB) and the subsequent measurement of the acetylated PAB excreted. Young male rats weighing around 100 gm. or mature rats averaging over 300 gm. were used in various experiments. Those selected for pantothenate deficiency were given a purified diet free of calcium pantothenate containing 73 per cent glucose, 18 per cent vitamin-free casein, 4 per cent corn oil (Mazola), 4 per cent Salts IV (6), 1 per cent cod liver oil, and the following vitamins[2] per 100 gm.: thiamine chloride 400 γ, riboflavin 800 γ, pyridoxine hydrochloride 400 γ, nicotinic acid 4000 γ, and choline chloride 100 mg. Control animals received 2.5 mg. of calcium pantothenate per 100 gm. of ration. For acetylation measurements, a larger series was run with 1 mg. of PAB given intraperitoneally per animal, and a smaller series with 2.5 mg. of PAB. The urine of the following 24 hours was analyzed for total and free PAB by the method of Bratton and Marshall (7). Bound PAB was calculated by the difference and expressed as per cent which was bound of the total excreted during the 24 hours. Blank runs showed no natural interference with the test at the concentrations used. Repeated PAB

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[1] This name has recently been used to identify the coenzyme by Kaplan and Lipmann (2).

[2] The crystalline vitamins used in this study were kindly supplied by Merck and Company, Inc., Rahway, New Jersey, and the glucose and corn oil by the Corn Industries Research Foundation, New York.

injections were made on the same animals with a minimum of 3 days between subsequent doses.

Response of deficient animals to the missing vitamin was determined on five rats which had been on the deficient diet for 2 months, on which consistently low acetylation values had been obtained. Individual amounts of 50 γ, 200 γ, 500 γ, or 1 mg. of calcium pantothenate were injected intraperitoneally in the same solution with the PAB dose. Following the 50 and 200 γ doses, the animals were allowed to return to the previous low acetylation values. These same animals were then given the 500 γ and 1 mg. doses. Finally, both deficient animals and controls were given 1 mg. injections of calcium pantothenate every 3 days. Pantothenic acid excretions were measured at various times on urine samples by microbiological assay, by the method of Hoag *et al.* (8).

Results

Table I summarizes the acetylation data on ten young rats initially weighing around 100 gm. each. An average of nearly 90 per cent of each PAB dose was excreted in 24 hours by these animals, regardless of dose size, supplement, or diet. Normal animals showed an average of 68.3 per cent acetylated PAB out of the total excreted. No marked change in acetylation was observed due to weight increase of the normals, although over the entire period of the experiment (over 4 months) the values showed a slight drift upward as their weight increased from around 100 gm. to well over 300 gm. (Fig. 1). Thus near the end of 4 months, the control animals bound 72.7 per cent PAB (Series 3, Table I), as compared to the over-all average of 68.3 per cent for the entire 4 months. Since the acetylations obtained after 24 hour fasting (74.9 per cent bound) were measured near the end of the experimental period, this average has been compared with the 72.7 per cent acetylation value.

The ability of the pantothenic acid-deficient animals to acetylate PAB was definitely below that of the control animals after 2 weeks on the diet, when the determinations were first made. The average acetylation of 50.0 per cent of 1 mg. doses then remained fairly constant, except for occasional radical variations. With 2.5 mg. of PAB, acetylations dropped to 36.7 per cent. With this dose, the addition of 3 per cent sodium acetate to the diet caused a significant increase in acetylation to 43.5 per cent. A similar rise with 3 per cent sodium acetate on 1 mg. doses of PAB was observed, but the great variation among the animals (s.E. = ±3.8 for a 7.3 per cent increase) leaves doubt as to its significance. Added acetate had no effect on the acetylation by normal animals, and 2.5 mg. of PAB were acetylated equally as well as 1 mg.

There was no apparent tendency for a continued fall in acetylation

TABLE I

Acetylation of p-Aminobenzoic Acid by Rats on Normal and Pantothenic Acid-Deficient Diets

Series No.	Treatment	PAB dose	Normal			Pantothenic acid-deficient			
			Trials	No. of animals	Acetylation*	Trials	No. of animals	Acetylation*	Decrease
		mg.			*per cent*			*per cent*	*per cent*
1	None	1	51	4	68.3 ± 0.78	54	6	50.0 ± 1.2	26.8
1	1% NaOAc diet	1	4	4	68.9 ± 2.9	6	6	50.0 ± 2.9	27.4
1	3% " "	1	3	3	70.8 ± 1.3	6	6	57.3 ± 3.8	19.1
2	None	2.5	3	3	66.3 ± 1.0	6	6	36.7 ± 1.6	44.6
2	3% NaOAc diet	2.5	6	3	65.1 ± 3.3	12	6	43.5 ± 1.9	33.1
3	None	1	9	3	72.7 ± 1.7				
3	24 hr. fasting	1	9	3	74.9 ± 1.1				
3	1 mg. Ca pantothenate injection	1	12	3	74.4 ± 0.64				

* Per cent bound ± standard error of the mean.

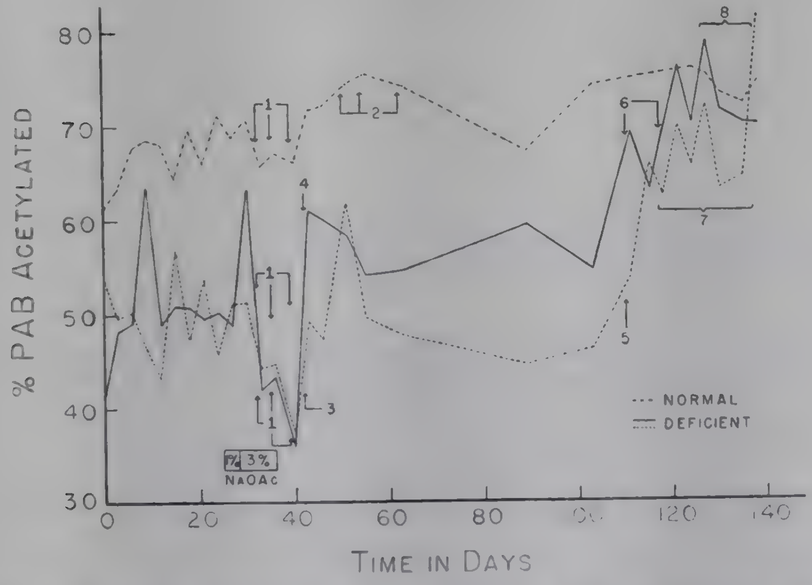

FIG. 1. The effect of pantothenic acid deficiency and of various treatments on acetylation of *p*-aminobenzoic acid by rats. PAB doses were 1 mg., except where indicated, and were administered 24 hours before the acetylation value plotted. *1*, 2.5 mg. of PAB dose; *2*, 24 hour fasting; *3*, 50 γ of calcium pantothenate intraperitoneally; *4*, 200 γ of calcium pantothenate intraperitoneally; *5*, 500 γ of calcium pantothenate intraperitoneally; *6*, 1 mg. of calcium pantothenate intraperitoneally; *7*, 1 mg. of calcium pantothenate intraperitoneally with each PAB dose given deficient animals; *8*, 1 mg. of calcium pantothenate intraperitoneally with each PAB dose given normal animals.

ability as the pantothenic acid deficiency progressed and the animals lost weight. This lower plateau is consistent with the observations of Olson and Kaplan,[3] who have found that the coenzyme A content of liver follows a somewhat similar course during pantothenic acid deficiency, falling to approximately one-fourth the normal concentration. The combined evidence thus indicates that, under the conditions of these experiments, the ability to acetylate PAB is a function of the coenzyme A concentration.

In Table II are shown the responses obtained when the deficient rats were injected with various levels of calcium pantothenate. Doses of 200 or 500 γ of the vitamin gave slight but inconsistent increases in acetylation. These were not significant on the few data available. 1 mg. doses, however, gave an immediate response to normal (69.5 per cent). This figure was not further increased by repeated doses. Normal animals injected with 1 mg. doses of calcium pantothenate acetylated 74.4 per cent, compared to the 72.7 per cent obtained in immediately preceding runs (Series 3, Table I). Fig. 1 summarizes the per cent acetylation by normal and deficient animals as a function of time, illustrating the marked changes which occurred when the various treatments were given. For clarification in demonstrating the response of deficient animals given various amounts of calcium pantothenate, two curves are shown for the rats on the pantothenic acid-deficient diet.

Table II

Response in Acetylation of 1 Mg. Doses of p-Aminobenzoic Acid by Pantothenic Acid-Deficient Rats Injected with Calcium Pantothenate

Ca pantothenate injected	Trials	No. of animals	Acetylation	
γ			*per cent**	*per cent of normal**
	54	6	50.0 ± 1.2	73
50	3	3	49.3 ± 1.4	72
200	3	3	61.1 ± 5.2	90
500	2	2	54.0 ± 1.6	79
mg.				
1	33	5	70.7 ± 1.1	104

* Per cent bound ± standard error of the mean.

† Compared to normal animals as 68.3 per cent (Table I).

Pantothenic acid excretion falls very rapidly within the 1st week on the deficient diet. Deficient animals showing low acetylation ordinarily excrete well under 1 γ per day. Following injection of the vitamin, up to 50 per cent of a given dose may be lost during the first 24 hours. Acetylations did not return to the low value characteristic of the deficiency for 2 weeks or more, in spite of no further source of pantothenate.

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[3] Olson, R. E., and Kaplan, N. O., in preparation.

DISCUSSION

The data in Table I show that the normal rats acetylated 18 per cent more of a 1 mg. dose of PAB than those on a pantothenic acid-deficient diet. This amounts to more than a 25 per cent decrease, due to the absence of the vitamin. With a larger dose of PAB (2.5 mg.) the deficient animals acetylated 29.6 per cent less than the controls, or a decrease of 44.6 per cent of the normal. Injection of 1 mg. of calcium pantothenate into deficient animals immediately raised their acetylating ability to normal, although single doses of 500 γ or less failed to give complete recovery in any animal. The extra 1 mg. to control animals already receiving a supposedly adequate level of the vitamin in the diet resulted in a slight increase in acetylation which may prove significant with more trials.

Apparently a considerable amount of pantothenic acid (up to 1 mg.) is needed at one time at the site of PAB acetylation (presumably the liver (9, 10)) for the immediate formation of sufficient coenzyme A to initiate a normal reaction after depletion. In view of the rapid loss of pantothenic acid in the urine following administration of injected doses, it would appear that the yield of coenzyme A is very small. Indeed, Novelli and Lipmann (11) have shown that bacteria yield but 4 to 8 per cent coenzyme A from a given amount of added pantothenate. On the other hand, animals retain remarkably good acetylation values (above 40 per cent), even after severe deficiency symptoms appear. This may be due in part to the relatively low PAB dose of 1 mg. given. Dose size unquestionably has much to do with the amount acetylated, as measured in the 24 hour urine. Bloomberg (9) reports that human subjects acetylate 100 per cent of a 25 mg. dose or less of PAB in 24 hours, while they bind only 70 to 75 per cent of a 100 mg. dose. Martin and Rennebaum (12) report values of but 3 per cent in 24 hour urines of rats given 1 mg. of sulfanilamide per gm. of body weight. Benigno (13) found rabbits to acetylate 76 per cent of a dose of 0.03 gm. of PAB per kilo of body weight in 12 hours, while Doisy and Westerfield (14), using 1 gm. doses in 2 kilo rabbits, found 30 to 40 per cent bound in 48 hour urines. Our control animals acetylated a 2.5 mg. dose not significantly different from 1 mg. (66.3 *versus* 68.3 per cent). In contrast, the deficient animals bound only 36.7 per cent as compared to 50.0 per cent of the 1 mg. dose, although with the larger dose the animals actually coupled twice as much acetate.

The addition of acetate to the diet did not produce any marked change in the ability of normal animals to acetylate either the 1 mg. or 2.5 mg. dose of PAB. Short periods of starvation were also without effect. However, with the deficient animals, the addition of acetate allowed considerable improvement. This was especially true at the higher dose of PAB when more acetate was needed. This effect may indicate that acetate formation is abnormal in pantothenic acid-deficient animals as well as the enzyme which makes use of it, whereas in normal animals the low doses of PAB used apparently do not cause any appreciable strain on the body system for acetate or coenzyme A formation or function.

By use of D-labeled sodium acetate, Bloch and Rittenberg (15) have now well demonstrated that acetate acts directly in acetylating PAB and is perhaps the sole directly contributing substance (*cf.* also (3)). Current workers, however, do not agree on the effect of added acetate on acetylation ability of an animal (12). Much of such disagreement may be due to dose size, as already indicated, to the animal used, or to the use of blood in preference to urine for analysis. Martin and Rennebaum (12) have shown the lack of correlation between blood and urine analyses. These factors may explain completely the failure of these authors (12) to show lowered acetylations in pantothenic acid-deficient rats. Their observed decreased acetylations in thiamine and in riboflavin deficiency may also be accounted for, since both these vitamins are known to function in enzymes which lead to formation of acetate. It is possible that, when large doses of PAB or sulfanilamide are given, the stress is primarily upon those systems which supply acetate, whereas with small doses failure of the acetylating mechanism may be shown more clearly. The present findings indicate that pantothenic acid functions in the acetylating mechanism of the rat, presumably through coenzyme A of Lipmann and collaborators.

SUMMARY

1. Normal rats were found to acetylate 70 per cent of the amount excreted in 24 hours after a 1 mg. or 2.5 mg. dose of *p*-aminobenzoic acid administered intraperitoneally.

2. Rats rendered pantothenic acid-deficient acetylated only 50 per cent of a 1 mg. dose and 37 per cent of a 2.5 mg. dose.

3. Simultaneous injection of 1 mg. of calcium pantothenate to deficient animals immediately returned their acetylation to normal.

4. The effect of added acetate, 24 hour fasting, and the size of the dose on the degree of acetylation have also been investigated.

The authors appreciate the suggestions and interest of Dr. F. Lipmann, Dr. N. O. Kaplan, and Mr. G. D. Novelli.

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SEPARATION AND CHARACTERIZATION OF CONALBUMIN*

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In addition to the extensively studied ovalbumins, the egg white of chickens contains, among other proteins, an entity designated by Longsworth, Cannan, and MacInnes (1) as conalbumin. According to these investigators, it comprises approximately 15 per cent of the whole egg white protein and migrates in this system as a single electrophoretic component over a wide series of pH values. However, when this component was separated by acidification of the albumin fraction of egg white to a pH below 4.0, the conalbumin showed two components upon electrophoretic and ultracentrifugal analysis.

It has been found possible to purify this protein by ethanol fractionation to give a product which is a single component both by electrophoresis and by sedimentation. Under the conditions we have used, this substance is separated as a flavoprotein, the dialyzable chromophore group of which is readily dissociated in solutions acid to the isoelectric point of the protein carrier.

EXPERIMENTAL

The general techniques involving the variables in the ethanol fractionation of protein systems (2) were used in this work. Fresh egg white served as the starting material. In all fractionations, the temperatures were maintained within 1° of the freezing point of the system; the pH measurements were carried out at 25° by means of a glass electrode. The progress of the fractionation was followed by electrophoretic analyses of the products in a barbiturate buffer of pH 8.6 and ionic strength of 0.1 at a constant potential gradient of between 6.0 to 6.4 volts per cm. Mobility determinations were carried out on 0.5 per cent solutions of the purified protein at constant potential gradients of approximately 4.5 volts per cm. in 0.1 ionic strength buffer solutions. In these solutions NaCl supplied 80 per

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cent of the ionic strength, while the buffer ion, barbiturate, glycine, cacodylate, or acetate, depending on the pH, supplied the remainder. Throughout this paper only the descending electrophoretic patterns are shown.

Sedimentation analyses[1] were carried out on 0.7 per cent solutions of the conalbumin in 0.15 M NaCl in the standard Svedberg oil-turbine ultracentrifuge at approximately 220,000 × gravity, with a schlieren optical system to record the position of boundaries as a function of time.

Absorption spectra were determined in a Beckman spectrophotometer. The samples for the absorption measurements were made up at 2 per cent protein concentration in 0.1 M NaCl. For each observation, 5 cc. portions of the protein solution previously adjusted to the desired pH were dialyzed for several days against repeated changes of 0.1 M NaCl which had been adjusted to pH 4.3 or 8.3 with dilute acid or base.

Results

Application of the conditions of the ethanol fractionation to chicken egg white for the purpose of separating conalbumin resulted in the accompanying fractionation scheme. The progress of a typical fractionation is shown in Fig. 1 by the electrophoretic patterns of the products obtained at the various steps of the scheme. The conalbumin is designated as C in the scheme and is analogous to the component of chicken egg white designated as C_2 by Bain and Deutsch (3). Table I gives the results of a typical fractionation in terms of purity of product and yield. It may be seen that the conalbumin has been isolated from egg white, in which it occurs as 16 per cent of the total proteins, and so purified that a product is obtained which is a single electrophoretic component under the conditions of analysis. From 1 liter of egg white, it is possible without any special attention to yield to obtain 2 gm. of this product. This amount may be increased, if desired, by further fractionation of Precipitate III. The purified preparations have a tendency to form a small amount of a viscous precipitate when the lyophilyzed products are dissolved, apparently a result of denaturation. Attempts to crystallize the conalbumin were unsuccessful.

The presence of flavin in conalbumin was suspected during the course of pH-mobility investigations when it was noticed that the solutions which were dialyzed at pH 6.0 or below lost their characteristic yellow color and became water-clear, while those samples dialyzed at pH 7.0 and above remained colored. Adjustment of the pH alone was not sufficient to bring about this change. It appeared that some dialyzable chromogen was associated with, or adsorbed on, the conalbumin at high pH and was

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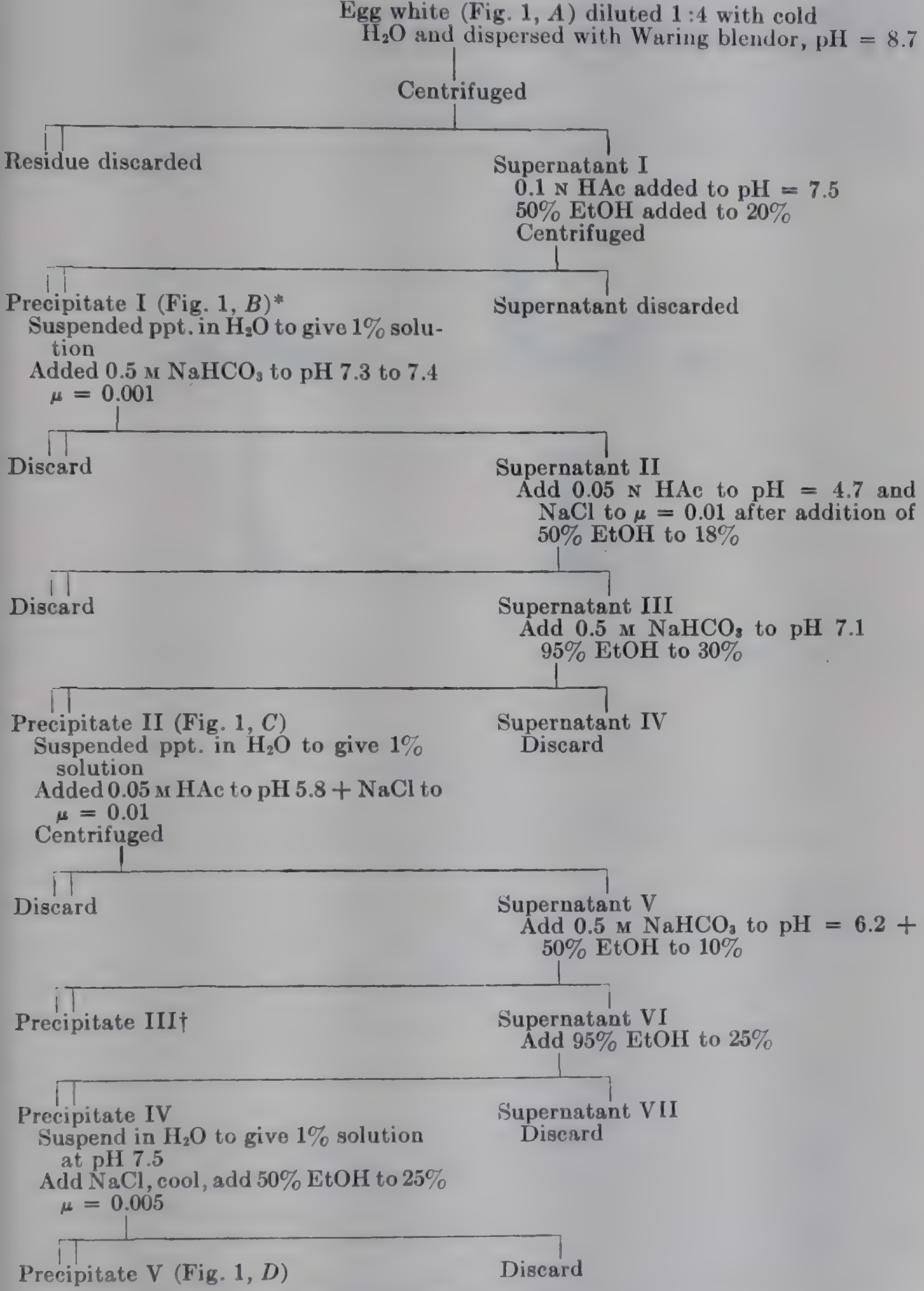
[1] All sedimentation experiments were carried out by Mr. E. M. Hanson of this Laboratory. His careful attention to this phase of the work has contributed much to our research.

Fractionation Scheme for Conalbumin



Egg white (Fig. 1, *A*) diluted 1:4 with cold H_2O and dispersed with Waring blendor, pH = 8.7

Centrifuged

Residue discarded

Supernatant I
0.1 N HAc added to pH = 7.5
50% EtOH added to 20%
Centrifuged

Precipitate I (Fig. 1, *B*)*
Suspended ppt. in H_2O to give 1% solution
Added 0.5 M $NaHCO_3$ to pH 7.3 to 7.4
$\mu = 0.001$

Supernatant discarded

Discard

Supernatant II
Add 0.05 N HAc to pH = 4.7 and NaCl to $\mu = 0.01$ after addition of 50% EtOH to 18%

Discard

Supernatant III
Add 0.5 M $NaHCO_3$ to pH 7.1
95% EtOH to 30%

Precipitate II (Fig. 1, *C*)
Suspended ppt. in H_2O to give 1% solution
Added 0.05 M HAc to pH 5.8 + NaCl to $\mu = 0.01$
Centrifuged

Supernatant IV
Discard

Discard

Supernatant V
Add 0.5 M $NaHCO_3$ to pH = 6.2 + 50% EtOH to 10%

Precipitate III†

Supernatant VI
Add 95% EtOH to 25%

Precipitate IV
Suspend in H_2O to give 1% solution at pH 7.5
Add NaCl, cool, add 50% EtOH to 25%
$\mu = 0.005$

Supernatant VII
Discard

Precipitate V (Fig. 1, *D*)

Discard

* All precipitates dried from the frozen state.

† Precipitate III may be reworked as Precipitate II to recover additional pure material as Precipitate V.

dissociated and dialyzed away below pH 6.0. Evidence for this view is presented in Fig. 2, where the absorption spectra of samples dialyzed at pH 4.3 and 8.3 are given. Curve 1, dialyzed at pH 8.3, shows a maximum at approximately 280 mμ characteristic of protein, and second and third maxima at 370 and 450 mμ respectively, which are presumably due to the chromogen. Curve 3, dialyzed at pH 4.3, on the other hand, shows only the maximum at 280 mμ corresponding to simple proteins. Curve 2, with a preliminary dialysis at pH 4.3, followed by a second dialysis at pH 8.3, resulted in a preparation showing only the 280 mμ maximum in the light-absorption curve.

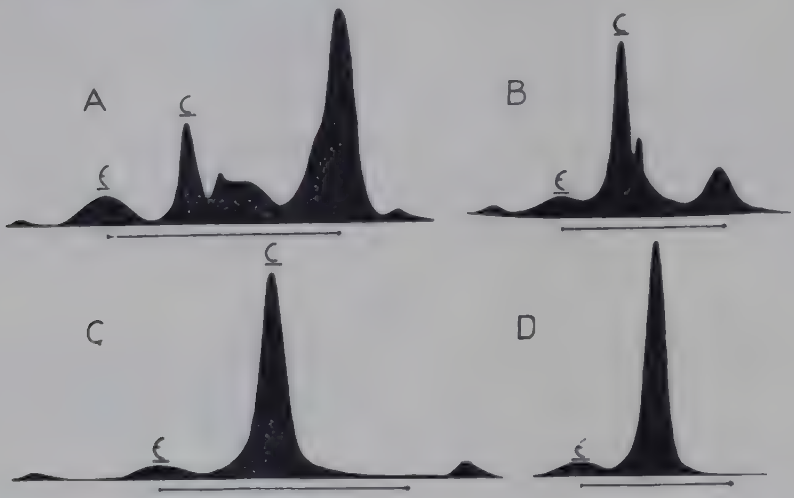


Fig. 1. Electrophoretic patterns showing progress of fractionation of conalbumin. Duration of experiment, *A*, 10,800 seconds; *B* and *D*, 7,200 seconds; *C*, 12,600 seconds.

Table I

Results of Typical Fractionation in Terms of Purity and Yield

	Total protein	Conalbumin	Yield of conalbumin
	gm.	*per cent*	*per cent*
Whole egg white (1 l.), Fig. 1, *A*	120	16	
Precipitate I,* Fig. 1, *B*	32	54	90
" II, " 1, *C*	12	91	58
" III	6.5	78	27
" IV	2.5	99–100	13
" V, Fig. 1, *D*	2.3	100	12

* *Cf.* the accompanying scheme.

The first two maxima of Curve 1, Fig. 2, correspond closely to those of riboflavin (4). Furthermore, acid solutions of the conalbumin showed a marked greenish fluorescence on irradiation with ultraviolet light. The addition of 3 volumes of methyl alcohol to an acid solution precipitated the protein, leaving the fluorescent pigment in solution. The absorption curve of this methanol supernatant solution is shown in Curve 4 of Fig. 2. The maxima agree well with those reported by Warburg and Christian (4) for riboflavin, and by Ball (5) for xanthine oxidase, a flavoprotein. Furthermore, the yellow color of the conalbumin preparations is discharged by addition of hydrosulfite to acid solutions.

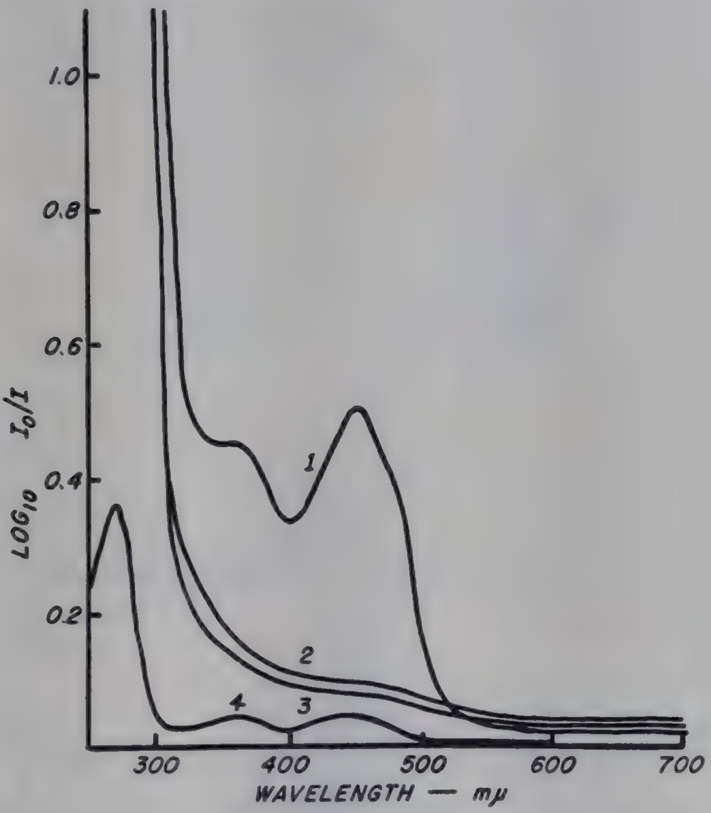


FIG. 2. Absorption curves of conalbumin preparations. Curve 1 represents a 2 per cent conalbumin solution dialyzed 72 hours at pH 8.3; Curve 2, the same solution dialyzed 36 hours at pH 4.3, then 36 hours at pH 8.3; Curve 3, the solution dialyzed 72 hours at pH 4.3; Curve 4, the supernatant resulting from addition of 3 volumes of MeOH to 2 per cent conalbumin solution at 38°.

A portion of the flavin attached to the conalbumin is lost during the fractionation. Thus, when a sample of Precipitate IV in the accompanying scheme was separated stepwise into two fractions at pH 6.0, the first at 11.5 per cent and the second at 25 per cent ethanol, it was found that Precipitate II contained only 75 per cent as much flavin per unit protein as Precipitate I, although both were single electrophoretic components at pH 8.6. Accordingly, we are unable to draw conclusions as to the molar ratio of flavin and protein in the native conalbumin, although the preparations which were examined spectrophotometrically always showed less than 1 mole of flavin per mole of protein. In this connection, Theorell (6) has concluded that the yellow enzyme contains 1 mole of flavin per mole of protein.

The isoelectric point of the conalbumin, as determined by pH-mobility studies (Fig. 3), is 6.1 at 0.1 ionic strength, not greatly different from the values of 5.8 and 6.0 found by Longsworth *et al.* (1) for the C_1 and C_2 components. However, we have found no evidence for more than one component in buffers at 0.1 ionic strength at any pH from 3.0 to 8.6. Indeed, as judged visually, the peaks were equally sharp and symmetrical throughout this pH range. However, when the conalbumin was subjected to electrophoretic analysis at a low ionic strength (0.01) and near the isoelectric point, several peaks appeared.[2] This result upon electrophoresis at a low ionic strength may be related to the amount of flavin associated with a given preparation and is being studied further. Immunochemical studies also suggest the presence of more than one protein.[3] When a conalbumin sample was dialyzed for 48 hours at pH 3.0 and then subjected to electrophoresis in a pH 5.2 buffer of 0.1 ionic strength, two components appeared. As seen from Fig. 4, conalbumin, which was dialyzed and studied electrophoretically at pH 5.2, showed the presence of only one component. These components have been designated according to the terminology used by

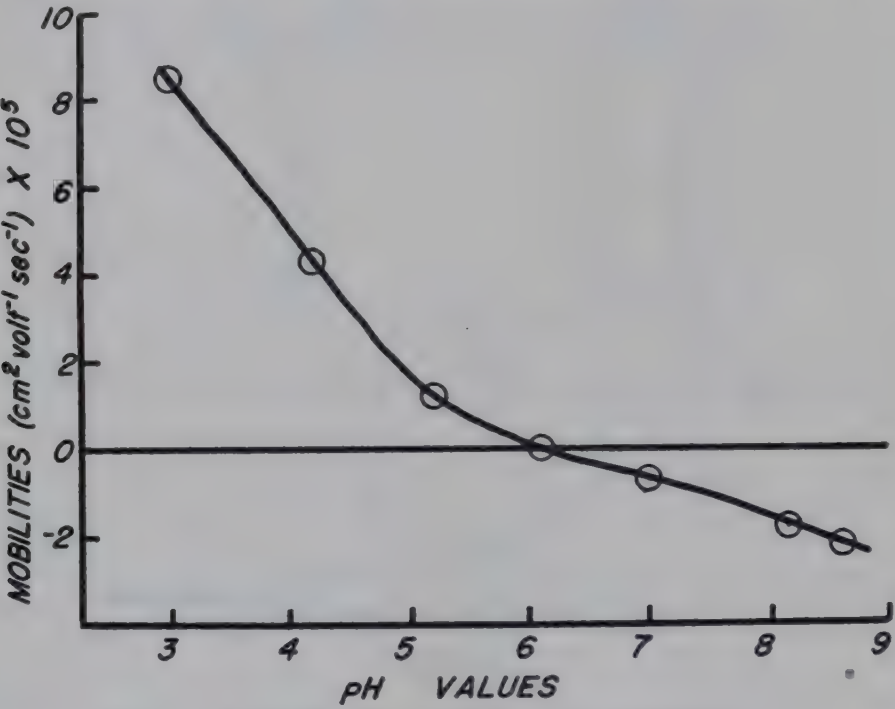


FIG. 3. pH-mobility curve of purified conalbumin

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[2] Alberty, R. A., and Anderson, E. A., unpublished experiments.

[3] Cohn, M., Wetter, L. R., and Deutsch, H. F., unpublished experiments.

Longsworth, Cannan, and MacInnes (1) for a similarly modified protein system and are not analogous to the native C_2 and C_1 conalbumin components of chicken egg white, as recorded by Bain and Deutsch (3).

Dialysis of the conalbumin at pH 4.5 against repeated changes of buffer for 5 days to remove the flavin gave a product which, upon electrophoretic study in buffers of 0.1 ionic strength, showed a single component with the same pH-mobility values in the region of its isoelectric point as did the flavoprotein.

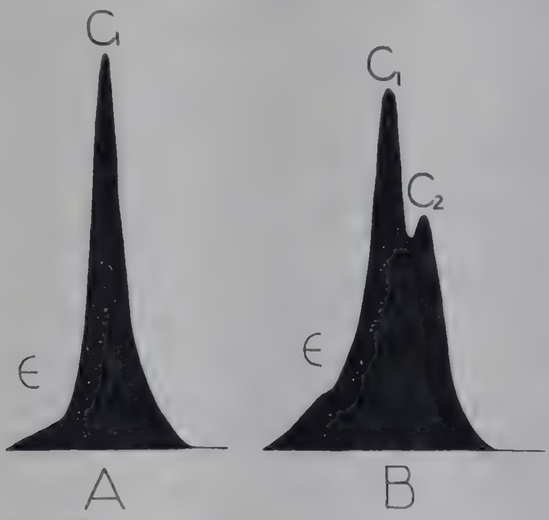

FIG. 4. Electrophoretic patterns of conalbumin at pH 5.2 in 0.1 ionic strength buffer. Duration of experiments, 10,800 seconds at a potential gradient of 3.8 volts per cm. *A*, dialyzed for 48 hours at pH 5.2 only; *B*, dialyzed at pH 3.0 at 0.15 ionic strength for 48 hours prior to 48 hours dialysis at pH 5.2.

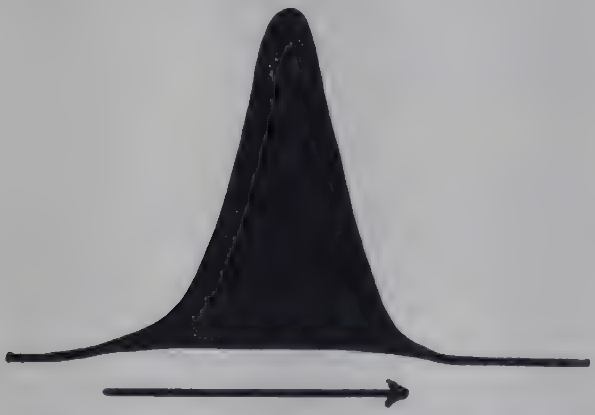

FIG. 5. Sedimentation pattern of purified conalbumin after 100 minutes at 220,000 times gravity.

It is of interest to note that the isoelectric point found by Ball (5) for xanthine oxidase was 6.2, as compared to 6.1 found for conalbumin. The conalbumin, however, showed no xanthine oxidase activity as tested by the

Thunberg technique. No other assays for enzyme systems requiring flavin were attempted.

In the ultracentrifuge, the conalbumin sedimented as a single component (Fig. 5) having a velocity of s_{20w} = 5.4 Svedberg units. In order to apply a criterion of homogeneity to a sedimentation diagram, several apparent diffusion constants were calculated from the data of velocity sedimentation experiments. An average apparent diffusion constant value of $D_{20w} = 8.4 \times 10^{-7}$ cm.^{2} per second was obtained, and there was no increase in the constant with time. Such a result indicates that the conalbumin is essentially monodisperse with regard to sedimentation properties. More detailed diffusion studies by Kegeles, Gosting, and Morris[4] indicated the presence of some inhomogeneous material and gave an average value for D_{20w} of 5.66×10^{-7} cm.^{2} per second. With the latter diffusion constant and a partial specific volume of 0.731, the value found for Warburg's yellow enzyme (7), a molecular weight of 87,000 may be calculated.

When a conalbumin preparation such as that shown in Fig. 4, *B*, which had been dialyzed at pH 3.0, was studied in the ultracentrifuge at pH 6.1, it sedimented as a single component. However, it did show the presence of small amounts of heavier inhomogeneous material analogous to the faster sedimenting component of the conalbumin preparations of Longsworth, Cannan, and MacInnes (1).

Nitrogen analyses of a well dialyzed conalbumin sample dried to constant weight *in vacuo* at 70° gave a value of 16.3 per cent.[5] No carbohydrate could be detected by the Molisch test in a conalbumin preparation from which flavin had been removed.

DISCUSSION

The presence of riboflavin in egg white has long been known. At least a part of this flavin appears to be associated with conalbumin, the protein whose separation from the egg white system by alcoholic fractionation methods is reported here. It appeared, however, that in these alcohol-containing systems a portion of the flavin associated with the conalbumin is lost during fractionation, and in this respect the product described should not be considered to be "native" conalbumin. The conalbumin preparations of Longsworth, Cannan, and MacInnes (1), which show the presence of two electrophoretic components, are not analogous to the native conalbumin which, in egg white, appears as a single electrophoretic component above pH 4.0. Dialysis of our electrophoretically homogeneous conalbumin preparations in the pH range used by the above workers in their separation of conalbumin effects a change in the protein which is characterized by the appearance of a second electrophoretic component (see Fig. 4, *B*). Electrophoretically homogeneous conalbumin shows a single component upon sedimentation analysis. Conalbumin dialyzed for 48 hours at pH 3.0 shows the presence of small amounts of faster sedimenting inhomogeneous material. However, by such treatment we were unable to produce the relatively large amounts of faster sedimenting material of approximately twice the molecular weight of the main component, as noted by Longsworth, Cannan, and MacInnes (1) for their conalbumin preparations.

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[4] Kegeles, G., Gosting, L. J., and Morris, M. S., unpublished data.

[5] We wish to thank Mr. L. R. Wetter for this determination.

A definite association of the flavin with the protein seems established by the failure of the chromophore to dialyze away from the protein at pH alkaline to the isoelectric point. However, the dissociation tendencies of the complex seem to be greater than those of other flavoproteins previously studied (8). Theorell (6) was able to study the electrophoretic properties of Warburg's yellow enzyme at pH values acid to the isoelectric point. In our experience with conalbumin, the flavin dissociates from the protein by dialysis under such conditions and we have been unable to make comparable electrophoretic observations. Theorell (6) also found that the isoelectric point of the yellow enzyme shifts approximately 0.5 pH unit upon removal of the flavin, while conalbumin showed no such shift.

SUMMARY

Conalbumin has been separated from the egg white proteins by application of aqueous, ethanol fractionation procedures. A product has been obtained which is essentially pure, as judged by its electrophoretic and sedimentation behavior. It has a molecular weight of approximately 87,000 and exhibits the properties of a flavoprotein. The flavin separates readily from conalbumin in solutions acid to its isoelectric point.

The authors wish to thank Dr. J. W. Williams for his interest during the course of this investigation.

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STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, PLASMA COPPER, AND PLASMA IRON IN NORMAL AND IN PYRIDOXINE-DEFICIENT SWINE*

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(Received for publication, September 19, 1947)

Previous communications from this laboratory (1–7) have demonstrated that swine maintained on a highly purified diet deficient in pyridoxine develop a severe anemia which is characterized by microcytosis, slight hypochromia, and an increase in polychromatophilia, reticulocytes, and nucleated red cells in the blood. There is a marked hyperferremia, normoblastic bone marrow hyperplasia, myelin degeneration in the peripheral nerves, and hemosiderosis in the liver, spleen, and bone marrow. Certain alterations in tryptophan metabolism, namely an increased excretion of xanthurenic acid, kynurenine, and urorosein, occur. The administration of pyridoxine is followed by a sharp reticulocyte response, mobilization of iron from the tissues, and rapid regeneration of blood with restoration of the normal size of erythrocytes. The anemia fails to respond to the administration of either iron or purified liver extract. Appropriate studies have failed to reveal evidence that the anemia is due to increased blood destruction and it has been concluded that it is due to a disturbance in erythropoiesis. The nature of this disturbance has not, however, been discovered.

The purpose of this report is to present data concerning certain aspects of the metabolism of porphyrin, copper, and iron in pyridoxine-deficient swine, which were gathered in an attempt to elucidate the mechanism by which a deficiency of pyridoxine produces anemia. The literature dealing with the relation of pyridoxine to erythropoiesis has recently been reviewed (8).

Materials and Methods

Full details of the experimental methods have been given elsewhere (9). For this study, which forms part of a larger one, forty-five weanling pigs, approximately 21 days of age, were used. Thirty-one animals were placed on the control diet and fourteen animals were fed the same diet as the

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* Aided by a grant for the study of the pathogenesis of the anemia of infection from the United States Public Health Service, and by grants for the study of hematology and nutrition from The Upjohn Company and Parke, Davis and Company.

controls except that pyridoxine was omitted. The results of observations on pigs with other types of deficiency will be reported later.

All animals from the day they were received were fed the basal diet consisting of Sheffield "new process" casein 26.1 per cent, sucrose 57.7 per cent, lard 11.0 per cent, salt mixture (swine Salt Mixture 3 (9)) 5.2 per cent. In addition they were given cod liver oil (Mead Johnson, 1800 units of vitamin A, 175 units of vitamin D per gm.), 0.5 gm. per kilo of body weight daily or Natola (Parke, Davis, 55,000 units of vitamin A, 11,000 units of vitamin D per gm.), 0.056 gm. per kilo of body weight per week. Vitamins were supplied in crystalline form in capsules and were administered orally three times a week. The quantities of crystalline vitamins were as follows (mg. per kilo of body weight daily): thiamine hydrochloride 0.25, riboflavin 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20, pantothenic acid 0.50, *p*-aminobenzoic acid 0.10, inositol 0.10, choline chloride 10.0.

Determinations of erythrocyte protoporphyrin were made by the method of Grinstein and Watson (10). Plasma copper was determined by the method of Cartwright, Jones, and Wintrobe (11). For the determinations of plasma iron the method of Kitzes, Elvehjem, and Schuette (12) as well as the method of Barkan and Walker (13) was used. Urinary coproporphyrin determinations were made by the method of Cartwright, Lauritsen, Jones, Merrill, and Wintrobe (14).

Results

Detailed hematologic and chemical data for each of the fourteen pyridoxine-deficient pigs are presented in Table I. In Table II the results of the chemical data are summarized. The amount of free protoporphyrin in the erythrocytes of the normal animals was 118 ± 43.4 γ per 100 ml. of red cells. In the pyridoxine-deficient group this was reduced to an average of 47 ± 13.6 γ. The coproporphyrin excretion in the urine in pyridoxine deficiency was not altered significantly from the normal of about 104 γ per 24 hours. The amount of copper in the plasma of the normal pigs was 206 ± 26.3 γ per cent. In the pyridoxine-deficient group the plasma copper was reduced to 160 ± 38.8 γ per cent. The plasma iron, on the other hand, was markedly increased, being on the average 468 ± 166.6 γ per cent as compared with the normal of 169 ± 38.8 γ per cent.

In Fig. 1 the data for erythrocyte protoporphyrin, plasma iron, and volume of packed red cells are presented as determined in a control animal and in a pyridoxine-deficient pig throughout the course of a 121 day experiment. The significant fact to be noted is that the amount of protoporphyrin in the erythrocytes dropped to the low value of 55 γ per 100 ml. of red cells on the 27th day of the experiment and thereafter remained constantly low. This change took place long before the development of significant anemia (90 days) and differed from the rise in plasma iron in that the latter increased gradually throughout the experiment. Similar results were obtained in four other animals, followed throughout the course of the deficiency.

TABLE I

Hematologic and Chemical Data for Fourteen Pyridoxine-Deficient Pigs

Pig No.	Red blood cells	Hb	Volume of packed red blood cells	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Erythrocyte protoporphyrin	Plasma copper	Plasma iron
	millions per c.mm.	*gm. per cent*	*ml. per 100 ml.*	*cu. micra*	*micromicrograms*	*per cent*	*γ per 100 ml. red blood cells*	*γ per cent*	*γ per cent*
Normal	8.86	14.7	47.0	53	17	32	118	206	169
9-05	9.70	11.5	44.0	45	12	26	23	183	295
9-06	6.02	5.8	22.0	37	10	26	48	137	578
9-07	4.95	3.7	15.0	31	8	25	47	117	440
9-08	7.00	8.4	32.0	45	10	26	45	102	490
9-20	2.60	3.1	11.5	44	12	27	41	127	392
9-21	6.48	7.6	27.0	42	12	28	79	113	436
9-22	6.50	5.9	20.5	32	9	29	56	201	730
9-23	9.21	9.3	37.0	40	10	25	27	177	376
9-39	7.03	7.3	26.5	38	10	28	63	218	788
9-40	5.85	8.3	28.5	49	14	29	38	143	635
9-41	5.70	7.8	27.0	47	14	29	65		921
9-42	5.35	8.6	28.0	52	16	31	59	237	618
10-20	5.70	8.1	26.0	46	14	31	64		438
10-21	7.40	8.7	29.5	40	12	29	50		466

TABLE II

Summary of Data

Determination	Group	No. of animals	No. of determinations	Mean ± S.D.
Erythrocyte protoporphyrin, γ *per 100 ml. red blood cells*	Control	31	208	118 ± 43.4
	Deficient	14	56	47 ± 13.6
Urinary coporporphyrin, γ *per 24 hrs.*	Control	4	10	104 ± 37.8
	Deficient	3	7	108 ± 36.3
Plasma copper, γ *per cent*	Control	23	79	206 ± 26.3
	Deficient	11	22	160 ± 38.8
Plasma iron, γ *per cent*	Control	30	230	169 ± 38.8
	Deficient	14	67	468 ±166.6

In Fig. 2 the results of intravenous therapy in a single animal with small doses of pyridoxal and pyridoxamine are presented. Within 24 hours after administration of pyridoxal the plasma iron dropped from 720 to 100 γ per cent. A maximum reticulocytosis of 18 per cent was reached on the

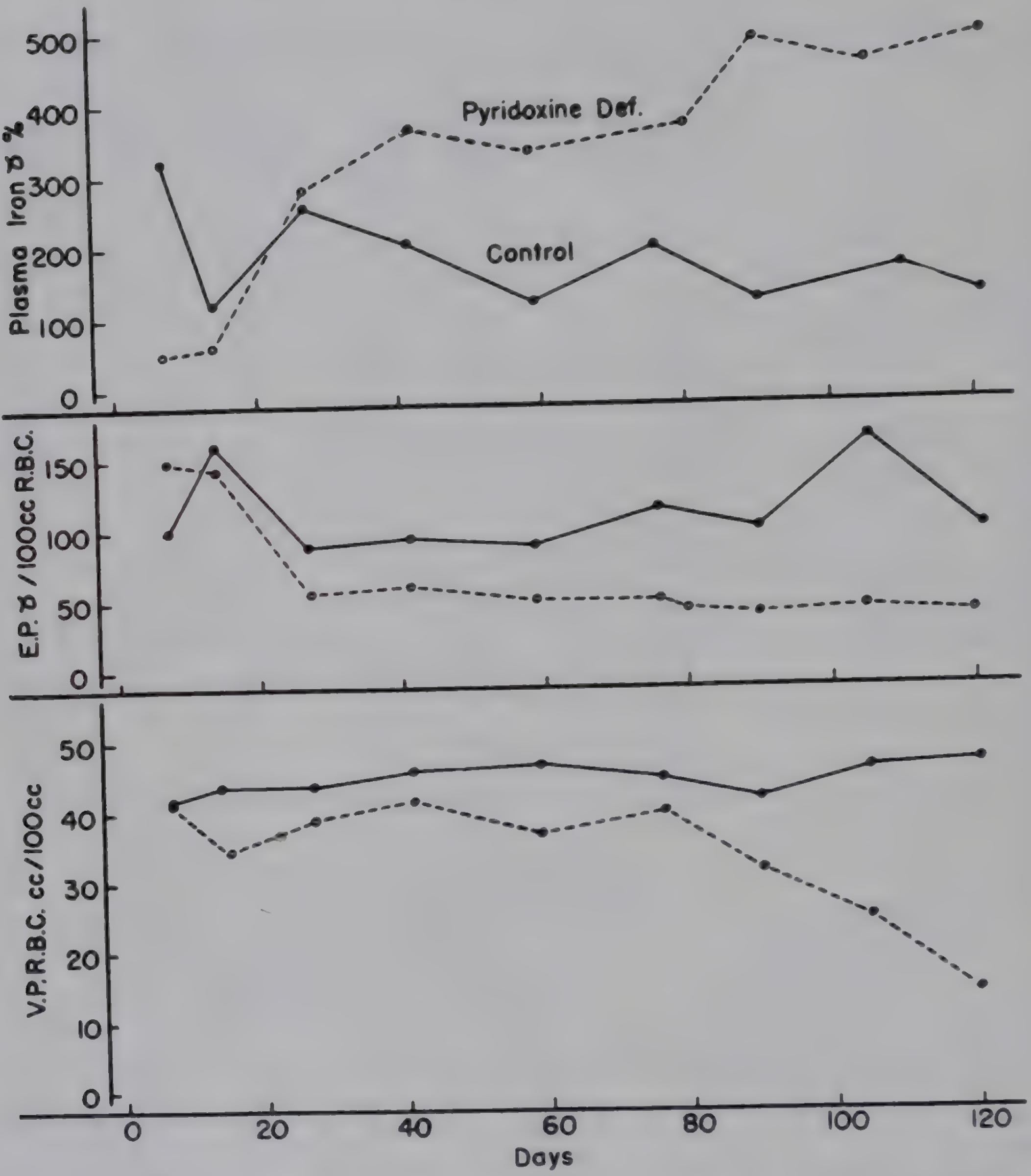

Fig. 1. Rise in plasma iron, reduction of free erythrocyte protoporphyrin, and development of anemia in a pig deficient in pyridoxine in comparison with a control animal.

3rd day of therapy. On the 5th day of treatment the erythrocyte protoporphyrin rose abruptly to 250 γ per 100 ml. of red cells from the previously low level of 55 γ. Following therapy the volume of packed red cells rose rapidly, the size of the erythrocytes increased to normal, the mean cor-

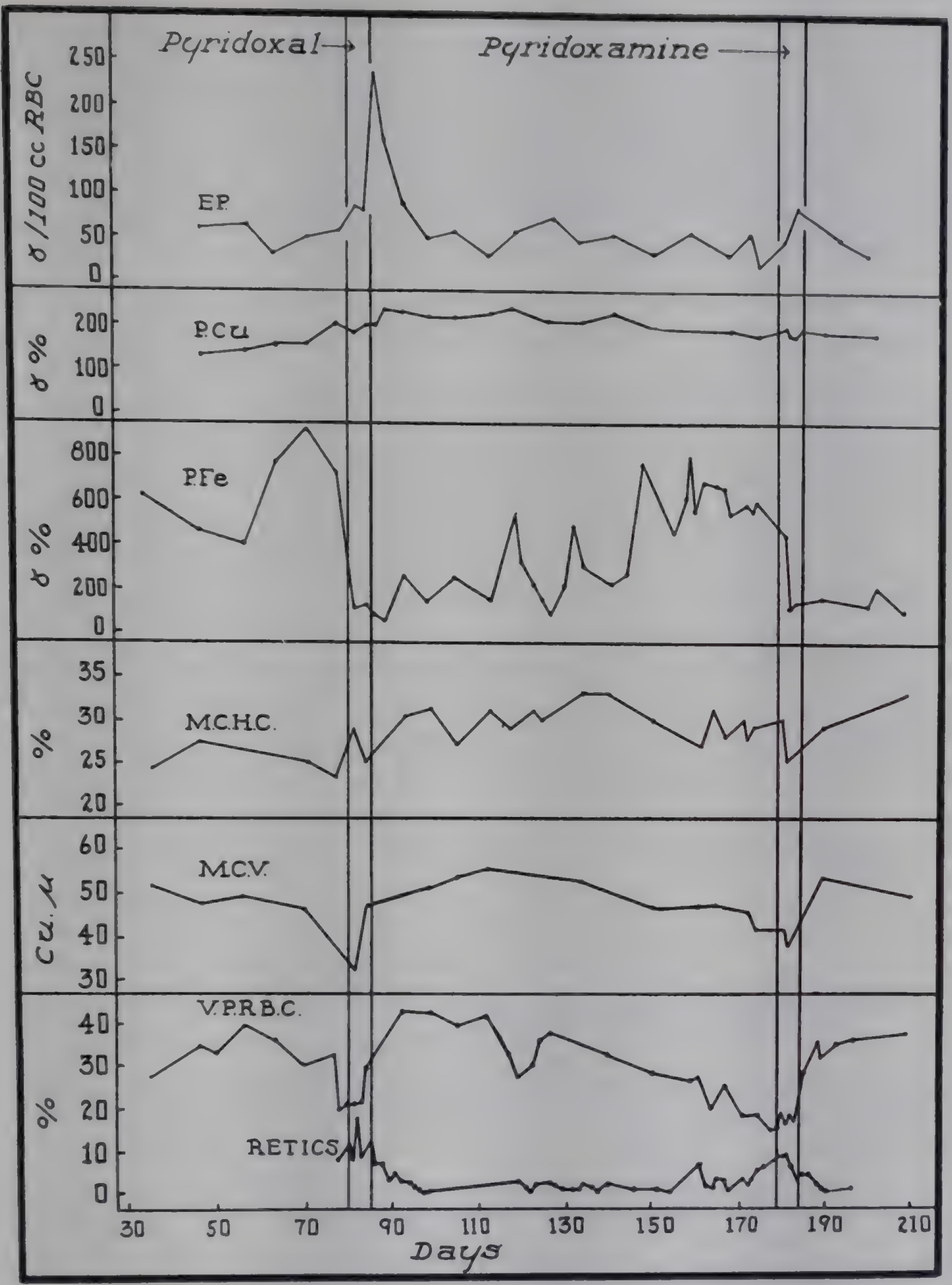

Fig. 2. Chemical and morphological changes in the blood of a pyridoxine-deficient pig following treatment with small doses of pyridoxal and pyridoxamine. The animal received 0.4 mg. per kilo of body weight on the 1st day of therapy and 0.2 mg. per kilo of body weight intravenously for 4 days thereafter.

puscular hemoglobin concentration returned to normal, and there was a slow gradual rise in plasma copper. 15 days after the cessation of therapy the erythrocyte protoporphyrin had returned to its previously low level, even though the plasma iron and copper levels were normal and anemia was not present. Over the course of the next 80 days a deficiency again developed and the animal was treated a second time with small doses of pyridoxamine. Again there was a reticulocytosis with restoration of the blood to normal, a rapid fall in plasma iron, and a slight rise in erythrocyte protoporphyrin. The experiment was discontinued on the 209th day, when the animal developed a secondary infection.

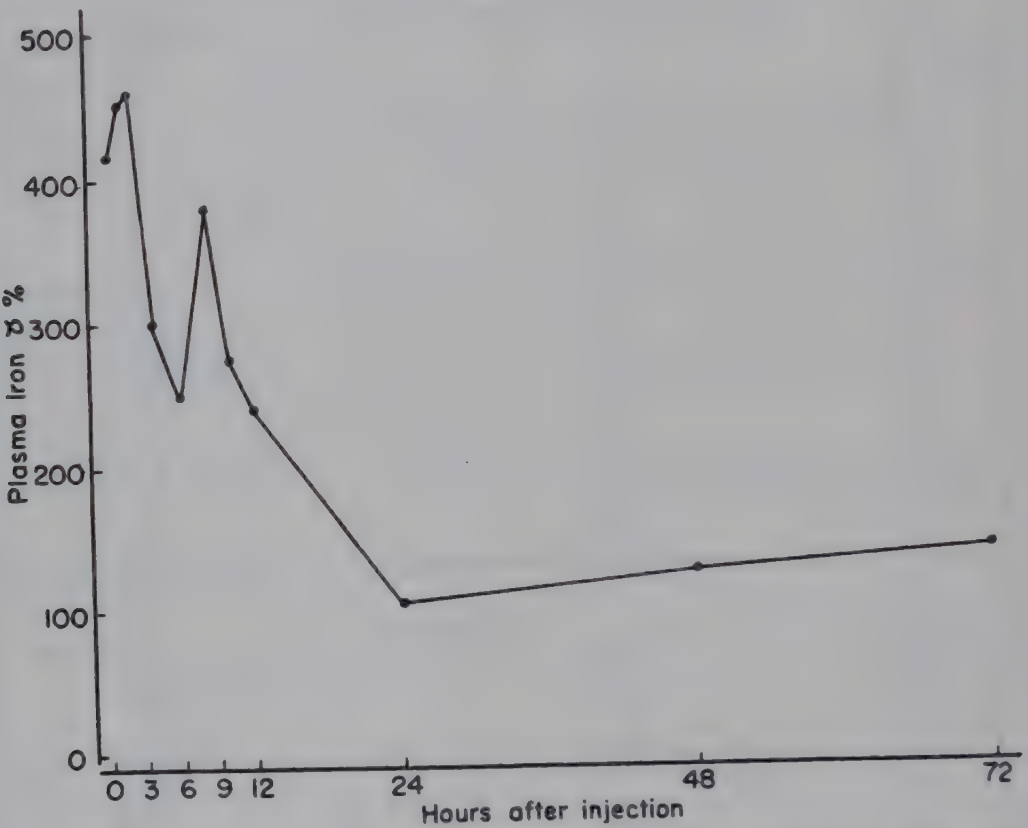

FIG. 3. The rapid fall in plasma iron in a pyridoxine-deficient pig following the intravenous injection of 0.4 mg. of pyridoxamine per kilo of body weight.

In order to determine the rapidity of the fall in plasma iron, determinations were made 1, 2, 3, 4, 6, 8, 10, 12, 24, and 48 hours following therapy. The results are presented in Fig. 3. As can be seen, the plasma iron began to decrease 2 hours after therapy and reached the lowest point within 24 hours.

DISCUSSION

The normal values for free erythrocyte protoporphyrin, plasma iron, and plasma copper in the pig differ significantly from the normal values in human subjects. In the pig the normal mean value for free erythrocyte protoporphyrin was found to be 118 ± 43.4 γ per 100 ml. of red cells (Table II). In human beings the normal erythrocyte protoporphyrin is less than 50 γ per 100 ml. of red cells (14, 15). The normal plasma copper and plasma iron for the pig have been found to be 206 ± 26.3 γ per cent and 169 ± 38.8 γ per cent, respectively. In human subjects the normal plasma copper is about 125 γ per cent (11) and the normal plasma iron about 126 γ per cent (8).

The reduction in the amount of free protoporphyrin in the erythrocytes of the pyridoxine-deficient animals suggests that the fundamental disturbance in erythropoiesis may be a failure to synthesize protoporphyrin. This is further suggested by the fact that the reduction in the free protoporphyrin appears to take place early in the course of the deficiency and precedes by a long period the development of significant anemia. This hypothesis also explains the hyperferremia and hemosiderosis of the tissues. Since protoporphyrin is not available, iron cannot unite with it to form heme, the plasma iron increases, and iron is stored in the tissues awaiting the time when protoporphyrin becomes available. When pyridoxine is supplied, the synthesis of protoporphyrin is accelerated and as the reaction protoporphyrin $+ \text{Fe}^{++} \rightarrow$ heme proceeds, the iron is mobilized from the serum and tissues, and there is an increase in hemoglobin with alleviation of the anemia. It has been demonstrated by Watson, Grinstein, and Hawkinson (15) that the free protoporphyrin in erythrocytes is protoporphyrin type III, No. 9, the protoporphyrin from which hemoglobin is synthesized. Evidence has recently been presented from our laboratory which indicates that the free protoporphyrin in the erythrocytes is a precursor rather than a degradation product of hemoglobin.[1]

At present one can only speculate concerning the manner in which pyridoxine may be related to protoporphyrin synthesis. It is known that the body does not depend upon a dietary source of pyrroles or their derivatives for the production of protoporphyrin. It has long been postulated that the pyrrole rings are synthesized from amino acids. Recently there has been direct evidence for this. Shemin and Rittenberg (16), using glycine labeled with isotopic nitrogen, demonstrated that glycine is a nitrogenous precursor of the protoporphyrin of hemoglobin in both the rat and man. It is now known that pyridoxine and its derivatives are intimately concerned with protein metabolism, especially decarboxylation and transamination of amino acids (17–21). Furthermore pyridoxine is known to be concerned with the metabolism of tryptophan, a pyrrole containing amino acid (8). It does not seem unlikely therefore that pyridoxine may be related to protoporphyrin synthesis through its relation to amino acid metabolism. If protoporphyrin synthesis is affected in pyridoxine deficiency, it is necessary to inquire whether other porphyrin-containing compounds such as catalase, the cytochromes, and indophenol oxidase are also affected. A deficiency of pyridoxine in the rat has not been found to cause a significant change in the catalase activity of the liver, kidney, and heart (22). However, the amount of protoporphyrin necessary for the maintenance of these enzyme systems, in comparison with the amount necessary for hemoglobin synthesis, is extremely small and perhaps these compounds would be affected last and not to a significant degree, since the reduction in free erythrocyte protoporphyrin is never complete. It should also be pointed out that in the rat uncomplicated pyridoxine deficiency results in little or no anemia.

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[1] Grinstein, M., Silva, J., and Wintrobe, M. M., to be published.

The slight reduction in plasma copper in pyridoxine deficiency is difficult to interpret. Very little is known about copper metabolism in various types of anemias. McKibbin *et al.* (23) found total blood copper at a low normal level in anemic pyridoxine-deficient dogs and observed that the level increased to normal during pyridoxine therapy.

We have pointed out previously that several similarities exist between pyridoxine deficiency anemia in swine and pernicious anemia in human beings; namely, hyperferremia, hemosiderosis of the tissues, hyperplasia of the bone marrow, and neurological lesions (2). In respect to the amount of free protoporphyrin in the erythrocytes the conditions are likewise similar, since in pernicious anemia the values tend to be in the low normal range (24).[2]

SUMMARY

1. Determinations of erythrocyte protoporphyrin have been made on thirty-one normal pigs and fourteen pyridoxine-deficient pigs. The mean plus or minus standard deviation for the normal group was 118 ± 43.4 γ per 100 ml. of red cells and for the pyridoxine-deficient group 47 ± 13.6 γ per 100 ml. of red cells.

2. Plasma iron determinations have been made on thirty normal pigs and fourteen pyridoxine-deficient pigs. The mean for the normal group was 169 ± 38.8 γ per cent and for the pyridoxine-deficient group 468 ± 166.6 γ per cent.

3. Plasma copper determinations have been made on twenty-three normal pigs and eleven pyridoxine-deficient pigs. The mean for the normal group was 206 ± 26.3 γ per cent and for the pyridoxine-deficient group 160 ± 38.8 γ per cent.

4. Urinary coproporphyrin excretion was measured in four normal pigs and three pyridoxine-deficient pigs. The mean for the normal group was 104 γ per 24 hours and for the pyridoxine-deficient group 108 γ per 24 hours.

[2] Cartwright, G. E., Huguley, C. M., and Wintrobe, M. M., to be published.

5. It is suggested that the fundamental disturbance in pyridoxine deficiency anemia in swine is a failure to synthesize protoporphyrin.

6. Certain similarities between pyridoxine deficiency in swine and pernicious anemia in human beings are described.

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THE EFFECT OF INSULIN UPON PYRUVATE UTILIZATION BY PIGEON MUSCLE

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The recent demonstration by Cori (1) that the hexokinase reaction is controlled by the interaction of insulin, anterior pituitary, and adrenal cortical hormones naturally raises the question as to whether the chemical action of insulin can be totally explained on the basis of this one effect. The evidence indicating that insulin is concerned more directly with oxidative reactions in the carbohydrate cycle is somewhat equivocal. However, there are experiments in the literature showing that insulin is concerned in oxidative reactions, particularly of pyruvate, which cannot be ignored. For example, Krebs and Eggleston (2) showed that the oxygen uptake of pigeon breast muscle mince was prolonged and therefore increased by insulin. This observation was amply confirmed by others (3–5). The observation of Stadie and Zapp (6) that pigeon breast muscle mince as customarily prepared contains no glucose and little or no glycogen and is dependent almost exclusively upon hexose phosphate for its metabolism would appear to exclude any possible rôle of hexokinase in experiments *in vitro*. In other words, this observation of Krebs and Eggleston shows that insulin may act on some enzyme system other than hexokinase. The inability to demonstrate an insulin action with muscle minces from other species (3–5) has made it difficult to assess the significance of this finding in relation to the general problem of insulin action.

Futher evidence must also be considered. Banga, Ochoa, and Peters (7) reported that insulin increases the utilization of pyruvate by minces of pigeon brain. Delrue and De Keyser (8) found that rabbits injected with pyruvate showed significantly lower blood pyruvate levels if simultaneously treated with insulin. However, the experiments of Bueding, Fazekas, Herrlich, and Himwich (9), showing that pyruvate blood levels in diabetic animals following the injection of glucose were low but could be significantly elevated by antecedent injection of insulin, have tended to support the concept that pyruvate formation rather than utilization was impaired in the diabetic state. But the evidence at hand does not warrant the conclusion that the two actions of insulin, *viz.* promotion of utilization as well as of formation, are mutually exclusive.

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More direct evidence that insulin is concerned with pyruvate utilization was reported by Rice and Evans (10). They equilibrated pigeon breast muscle minces with and without insulin in a preliminary period of 80 minutes and then added pyruvate. They demonstrated a significant effect of insulin upon the pyruvate utilization during the final period of 25 minutes, and concluded that their experiments demonstrated "a direct *in vitro* association between the action of insulin and the oxidation of a carbohydrate substrate, namely, pyruvic acid." The importance of this observation in relation to the general problem of insulin action led us to perform a series of experiments similar to those of Rice and Evans, the results of which are reported here.

Methods

The pigeons were fed *ad libitum* and killed by decapitation. The breast feathers were removed and the bird placed in ice water for 5 minutes. The breast muscle was excised and a Latapie mince prepared in the cold room. Suitable amounts of mince, usually about 250 mg., were weighed on a torsion balance and placed in Warburg vessels containing the medium previously cooled to 0°. The medium consisted of 3 parts of a phosphate-saline solution and 1 part of boiled pigeon breast muscle extract. The phosphate-saline solution had the following composition: 0.040 M sodium phosphate, 0.005 M KCl, 0.002 M $MgCl_2$, and NaCl to give an osmolarity of 0.300 M. The pH of the medium varied in the different experiments. The gas phase was 100 per cent oxygen. CO_2 was absorbed by an alkali filter paper inset in the center well. After temperature equilibration in the water bath, oxygen uptake was determined in the conventional way. The experiments were terminated by the addition of trichloroacetic acid to give a final concentration of 7 per cent. Pyruvate was determined on the filtrate by the method of Friedemann and Haugen (11).

Several kinds of insulin were used: an amorphous preparation and a crystalline zinc insulin, furnished by Eli Lilly and Company. In addition, insulin was prepared from "iletin" by isoelectric precipitation to remove phenol and glycerol present in this preparation. In some cases three precipitations were carried out.

EXPERIMENTAL

Latapie Minces—A large number of experiments, essentially similar to those of Rice and Evans (10), were performed. They are presented in Tables I and II. The vessels containing the mince were equilibrated for a preliminary period of 30 to 80 minutes with and without insulin. Known amounts of pyruvate (usually 30 micromoles) were then added from rotating side sacs and the oxygen uptake determined for the final period of 35 to 60 minutes. Each experiment was terminated by the addition of trichloroacetic acid and the final pyruvate determined. Pyruvate utilization was calculated as the difference of the initial and final pyruvate. In each experiment three to six separate samples with and without insulin were set up. Only the means of the results of each experiment are reported.

TABLE I

Effect of Insulin on Oxygen Uptake and Pyruvate Utilization of Pigeon Breast Muscle Mince (First Series)

Medium, phosphate-saline, pH 6.5; insulin, 1 unit per ml.; t = 38°. Each figure represents a mean of three or six determinations. The results are expressed in micromoles per 0.2 gm. per 45 minutes.

Experiment No.	Time of equilibration		Oxygen uptake		Pyruvate utilization	
	Initial	Final	Control	Increase with insulin	Control	Increase with insulin
	min.	*min.*				
1	80	60	4.0	7.0 ± 0.2	5.2	3.1 ± 2.2
2	80	60	12.1	3.7 ± 1.2	5.4	2.6 ± 0.4
3	80	50	(5.0)	(2.7 ± 0.4)	(1.2)	(0.7 ± 0.3)
4	80	50	(4.1)	(3.4 ± 0.9)	(2.5)	(0.7 ± 0.3)
5	30	35	22.0	−2.3 ± 1.0	19.2	5.7 ± 2.2
6	60	45	20.8	−1.0 ± 1.7	12.0	2.5 ± 0.9
7	60	45	13.2	4.9 ± 4.5	8.0	5.0 ± 1.0
8	60	45	15.9	3.4 ± 3.9	6.8	3.2 ± 1.2
9 (pH 7.2)	80	45	31.5	7.0 ± 3.1	18.7	4.1 ± 1.5
10 (" 7.2)	80	45	15.3	2.4 ± 2.7	7.2	5.9 ± 1.4
11 (" 7.2)	80	45	17.8	2.3 ± 1.5	10.5	2.5 ± 2.9
12 (" 7.8)	60	45	22.9	−2.0 ± 3.4	15.3	−0.6 ± 2.6
13 (" 7.8)	60	45	20.1	3.5 ± 1.9	9.8	4.2 ± 1.1
Mean.......................			17.8	+2.6 ± 0.98	10.7	+3.5 ± 0.55
Increase, %.................				+15 ± 5.5		+33 ± 5.1
t.........................				2.7		6.5

The results of Experiments 3 and 4 are excluded from the means on the basis of too little pyruvate utilization.

In this and subsequent tables the deviation measure is the standard error of the mean (S.E.M.). For the individual experiments it is calculated by the equation $\text{S.E.M.} = \sqrt{\text{S.E.M.}^2_{\text{insulin}} + \text{S.E.M.}^2_{\text{no insulin}}}$. For the entire series $\text{S.E.M.} = \sqrt{\Sigma d^2/(n(n-1))}$.

In Table I are given the data from the first series of experiments. Although there is a considerable variation in the results, there is a statistically significant effect of insulin both on the oxygen uptake and on the utilization of pyruvate. A second series of experiments was carried out some 6 months later and is reported in Table II. The insulin effects are smaller but still statistically significant.

Experiments with Slices—Slices of pigeon breast muscle, 0.5 mm. thick, were used in the same type of experiments as with the minces. After a preliminary period of 60 minutes, 30 micromoles of pyruvate were added and the oxygen uptake and pyruvate utilization were determined over a period of 60 minutes. The results are given in Table III. There were in all cases a greater oxygen uptake and pyruvate utilization in the presence of insulin. The effect is smaller than that observed with minces, but in the mean the effect of insulin is significant ($t = 3.6$).

TABLE II

Effect of Insulin on Oxygen Uptake and Pyruvate Utilization of Pigeon Breast Muscle Mince (Second Series)

Medium, phosphate-saline, pH 6.5; insulin, 1 unit per ml.; $t = 38°$; 60 minutes initial period; 45 minutes final period with pyruvate. Each figure represents the mean of three or six determinations. The results are expressed in micromoles per 0.2 gm. per 45 minutes.

Experiment No.	Oxygen uptake		Pyruvate utilization	
	Control	Increase with insulin	Control	Increase with insulin
1	22.5	1.8 ± 0.9	7.6	1.0 ± 0.3
2	24.6	2.8 ± 1.1	9.3	1.2 ± 0.6
3	23.0	3.6 ± 1.3	10.5	1.9 ± 0.7
4	21.7	0 ± 2.6	11.6	−0.6 ± 1.0
5	22.0	2.3 ± 1.4	8.6	1.0 ± 1.1
6	20.8	−1.0 ± 0.7	10.8	1.0 ± 0.2
7	13.4	1.9 ± 0.6	5.9	1.8 ± 0.2
8	17.2	0.7 ± 0.3	12.4	−0.7 ± 1.4
9	18.4	−0.4 ± 0.3	9.7	0.1 ± 0.3
10	13.3	2.6 ± 1.1	6.7	1.0 ± 0.8
11	15.8	1.3 ± 1.9	10.8	0.3 ± 2.1
12	13.6	0.2 ± 2.4	5.6	−0.3 ± 2.1
13	15.6	1.8 ± 1.1	8.0	1.6 ± 0.9
14	13.4	2.2 ± 1.2	6.2	1.2 ± 0.5
Mean	18.3	+1.4 ± 0.36	8.8	+0.8 ± 0.23
Increase,%		+8 ± 2.0		+9 ± 2.6
t		4.0		3.7

Miscellaneous Experiments—With pigeon muscle mince, the effect of several factors has been studied: (1) the pH of the medium, (2) the necessity of the presence of insulin in the preliminary period, and (3) the time of addition of pyruvate. Because of our inability to reproduce the effect of insulin in all experiments it is difficult to draw conclusions from the experiments designed to study the factors enumerated above.

pH—Essentially similar results were obtained over the range of pH 6.5 to 7.8.

Types of Insulin—Similar effects of insulin have been obtained with three types of insulin, *viz.* Lilly's amorphous insulin, Lilly's crystalline zinc insulin, and insulin prepared from "iletin" as described under "Methods."

Concentration of Insulin—In most of the experiments the concentration of insulin was 1 unit per ml. (1 mg. = 22 units). However, no difference in effect was obtained by varying the insulin from 1 to 6 units per ml.

TABLE III

Effect of Insulin on Pyruvate Utilization of Pigeon Breast Muscle Slices

Medium, phosphate-saline, pH 6.5; insulin, 1 unit per ml. Each figure represents the mean of the results from three samples of tissue. The results are expressed in micromoles per 0.4 gm. per 45 minutes.

Bird No.	Oxygen uptake		Pyruvate utilization	
	Control	Increase with insulin	Control	Increase with insulin
1	29.5	1.1 ± 3.1	16.0	0.3 ± 1.3
2	18.0	2.4 ± 2.3	7.9	1.3 ± 0.4
3	27.4	4.1 ± 1.0	14.1	2.1 ± 0.4
4	23.7	1.8 ± 1.9	14.1	2.4 ± 1.8
5	29.0	0.4 ± 2.7	15.2	0.9 ± 2.0
Mean............	25.5	2.0 ± 0.63	13.5	1.4 ± 0.38
Insulin, %.......		7 ± 2.9		10 ± 2.8
t................		2.4		3.6

Necessity of Fore Period—In about ten experiments, pyruvate was added during the preparation at 0° or after the brief (10 minute) period required for temperature equilibration at 38°. In other words, the fore period was eliminated. Insulin effects were obtained which in some instances were as great as those observed with the prolonged fore period.

Omission of Insulin in Fore Period—Positive effects of insulin on pyruvate utilization were obtained when the insulin together with the pyruvate was added at the end of the fore period. As noted previously, however, the results of the experiments in these last two cases were too variable to permit a conclusion as to whether a fore period with insulin present increases significantly the effect of insulin.

Experiments with Muscle Homogenates—The effect of more complete disintegration of the tissue was studied by the use of coarse homogenates of muscle. Such preparations would be intermediate between minces and extracts. About 5 gm. of Latapie mince were ground lightly in a mortar with 20 ml. of phosphate-saline (pH 6.5 to 7.2) plus 10 ml. of boiled muscle juice. After 15 minutes at 0° the suspension was filtered through gauze and the filtrate used. The oxygen uptake and pyruvate utilization of 60 samples from ten pigeons were determined as in the experiments with minces. There was a good utilization of pyruvate in all cases (mean 11.4 micromoles of pyruvate per sample per 45 minutes) but there was no significant effect of insulin (mean increase with insulin, +0.5 ± 0.2 micromoles). In two experiments, minces and homogenates from the same muscle were compared. Significant insulin effects on pyruvate utilization were obtained with the minces, but none with the homogenates.

TABLE IV

Effect of Insulin on Oxygen Uptake and Pyruvate Utilization of Pigeon Breast Muscle Mince in Presence of Malonate and Fumarate or Malate

Medium, phosphate-saline, pH 6.5; insulin, 1 unit per ml.; t = 38°; 60 minutes initial period; 45 minutes final period. Addition in final period, malonate 60 micromoles (0.015 M), pyruvate, fumarate, and malate as indicated in the table. Each figure is the mean of three or six determinations.

Experiment No.	Addition		Pyruvate added	Oxygen uptake, micromoles per 0.2 gm. per 45 min.		Pyruvate utilization, micromoles per 0.2 gm. per 45 min.	
				Control	Increase with insulin	Control	Increase with insulin
		micromoles	*micromoles*				
1	Fumarate	16	15	9.7	5.0 ± 1.5	6.2	2.4 ± 0.6
2	"	16	15	16.7	−1.2 ± 1.1	9.0	−0.3 ± 0.6
3	"	16	15	11.0	−0.4 ± 0.8	6.6	0.4 ± 0.2
4	"	60	30			8.2	2.0 ± 0.8
5	"	60	30	16.0	0.2 ± 0.6	6.2	0.7 ± 0.8
6	Malate	60	30	18.0	1.4 ± 1.1	14.0	0.3 ± 0.7
7	"	60	30	4.2	5.7 ± 0.9	8.9	1.3 ± 0.6
8	"	60	30	16.0	1.1 ± 1.6	11.0	1.3 ± 0.6
9	"	60	30	12.8	0.2 ± 0.9	6.0	−0.3 ± 0.8
Mean				13.1	+1.5 ± 0.89	8.5	+0.9 ± 0.32
Increase, %					+11 ± 6.8		+11 ± 3.6
t					1.6		3.1

Experiments with Malonate—Pyruvate is presumably oxidized mainly through the tricarboxylic acid cycle, initiated by condensation with oxalacetate. The cycle is inhibited at the succinate stage by malonate, preventing further oxidation of pyruvate. However, if fumarate, malate, or oxalacetate is added, pyruvate oxidation can again occur. All three of these substances have been used in experiments in which the effect of insulin on pyruvate oxidation has been studied. The experiments with fumarate and malate are given in Table IV.

The experiments were similar to those without malonate. Mince was equilibrated in phosphate-saline for 1 hour; 60 micromoles of malonate (0.015 M), 30 micromoles of pyruvate, and 60 micromoles of malate (or fumarate) were then added. The oxygen uptake and the pyruvate utilization were measured for the subsequent 30 or 45 minutes. If malonate was present in the preliminary period, there was little or no utilization of pyruvate in the subsequent period. There is considerable variation in the magnitude of the insulin effect. In about half of the experiments there was no significant effect of insulin, but the effects in the remaining are sufficient to make the mean value of all the experiments significantly different from zero. The magnitude of the effect is the same as that of our second series of experiments without malonate. Experiments were also done in which pyruvate plus oxalacetate was added in the presence of malonate.

TABLE V

Effect of Insulin on the Pyruvate Utilization of Pigeon Breast Muscle Mince in Presence of Malonate and Oxalacetate

Medium, phosphate-saline, pH 7.2; insulin, 1 unit per ml.; $t = 38°$; 60 minutes preliminary period; 45 minutes final period. Addition in final period, sodium malonate 60 micromoles (0.015 M), sodium pyruvate 30 micromoles (0.0075 M), and sodium oxalacetate as given in the table. 250 ± 5 mg. of tissue in each vessel.

Experiment No.	Insulin	Oxalacetate added	Δ oxalacetate		Δ pyruvate	
				Mean		Mean
		micromoles	*micromoles*	*micromoles*	*micromoles*	*micromoles*
1	0	22.3	−21.5		−7.1	
			−22.0	−21.8	−5.3	−6.2
	+	22.3	−21.9		−5.4	
			−22.0	−22.0	+1.9	−1.8
2	0	22.3	−21.2		−4.8	
			−21.9	−21.6	−4.0	−4.4
	+	22.3	−19.5		−8.7	
			−21.9	−20.7	−2.2	−5.5
3	0	25.5	−25.5		−0.5	
			−25.5		+6.7	
			−25.5	−25.5	0.0	+2.1
	+	25.5	−25.5		+2.5	
			−25.5		+3.5	
			−25.5	−25.5	+3.5	+3.2

After equilibration of the tissue with or without insulin in a fore period of 60 minutes, oxalacetate, pyruvate, and malonate were tipped into the vessels and the reaction allowed to proceed for 45 minutes more. The changes of the added substrates were determined as follows: (1) Δ oxalacetate was calculated from the initial and final oxalacetate, the latter being determined on trichloroacetic acid filtrates by the aniline citrate method (12). (2) The final total pyruvate + oxalacetate was determined by the 2,4-dinitrophenylhydrazine method of Friedemann and Haugen (11). The pyruvate could then be calculated. The results, presented in Table V, show (1) a rapid disappearance of oxalacetate, (2) a net decrease of pyruvate in two out of three experiments, (3) no demonstrable effect of insulin upon pyruvate utilization. In this respect the experiment differs from those with malate or fumarate, in which significant effects of insulin were observed in more than half of the experiments.

Formation of Acetate from Pyruvate

It has been demonstrated (13) that the reaction pyruvate = acetate + CO_2, occurs in muscle tissue. It is therefore conceivable that in the absence of added insulin pyruvate utilization in part stops at the acetate stage, but with insulin the utilization was more complete. The hypothesis was tested by determining acetate formation from pyruvate in the presence of pigeon muscle mince. At the end of the respiratory period a protein-free filtrate was prepared by the Somogyi method. The filtrate was acidified and 2,4-dinitrophenylhydrazine added. The precipitate containing the pyruvate was filtered off and the filtrate, after acidification to pH 3, was steam-distilled in a specially constructed distilling flask. The distillate was titrated against alkali with a micro burette. Control determinations showed that 1 micromole of acetic acid in the presence of amounts of pyruvate comparable to those used in the experiments could be recovered with an accuracy of about ±5 per cent.

Very little acetate was found in any of the experiments. The pyruvate utilization varied from 8 to 13 micromoles per 0.2 gm. of muscle, and the acetate formation from 0 to 1 micromole per 0.2 gm. Insulin in no way influenced the results; so that the hypothesis under discussion is unsupported.

Experiments with Rat Muscle Minces—To test whether the effect of insulin found in pigeon breast muscle mince is also demonstrable in mammalian tissue, scissors minces of rat pectoral muscle were prepared and experiments identical to those with pigeon breast muscle were carried out. Both normal rats and rats made diabetic by the injection of alloxan were used. In 72 samples from eight normal and four diabetic rats the mean pyruvate utilization was 3.2 ± 0.3 micromoles per 0.2 gm. per 45 minutes.

This rate is much lower than that observed with pigeon breast muscle (approximately 15 to 20 micromoles per 0.2 gm. per 45 minutes). However, no significant effects of insulin were observed.

Two experiments with human muscle were carried out. The pyruvate utilization by this tissue was so small that no conclusions could be drawn.

DISCUSSION

The objective evaluation of the data reported in this paper is beset with some difficulties. In view of the large number of experiments done (none being omitted), particularly those given in Tables I and II, the authors are inclined to give considerable weight to the statistical analysis and to the conclusions permissible therefrom. Since the experiments reported include all those performed, the fact that the mean increase of pyruvate utilization observed is statistically highly significant (the probability of difference arising by chance is less than 0.001) weighs heavily in regarding the effect of insulin upon pyruvate utilization as being real. On the other hand, our inability to control the system so as to give positive effects in all instances compels us to reserve a final opinion until other supportive evidence is available. For despite our best efforts, no or slightly negative effects of insulin were obtained in approximately one-third of the experiments.

The experiments in which malonate was present together with malate or fumarate, despite failures in half the cases, constitute, in our minds, additional evidence for the reality of the insulin effect on pyruvate utilization ($t = 3.1$, $P = 0.02$). Although there is insufficient evidence to formulate an opinion as to their full significance, the experiments would indicate that insulin is concerned in accelerating the condensation of pyruvate with the 4-carbon dicarboxylic acids, the initiating step in the Krebs cycle. Our failure to demonstrate an insulin effect when oxalacetate and pyruvate are equilibrated with muscle mince in the presence of malonate is contrary to the experience of Rice and Evans and not in accordance with the anticipations from our results with fumarate or malate. However, the unusual rapidity of the reaction as indicated by the rapid disappearance of oxalacetate might conceivably be unfavorable for the demonstration of an accelerating action of insulin.

The finding of a positive effect of insulin on pyruvate utilization with slices of pigeon breast muscle ($t = 3.6$, $P = 0.02$) is of importance. However, our complete failure in a large number of experiments to demonstrate any effect of insulin whatever upon coarse homogenates of muscle adds considerable confusion to the problem. In all cases, these homogenates utilized pyruvate vigorously and it is difficult, at this moment, to formulate any hypothesis to explain the difference of behavior of the homogenates from the minces. This is particularly puzzling when it is remembered that in experiments with the same bird positive effects were found with minces and none with the homogenates.

The prevailing opinion that experiments with pigeon muscle mince have little significance so far as the general problem of the chemical action of insulin is concerned has not been dissipated by our present experiments, for we failed completely to demonstrate any insulin effect upon pyruvate utilization by rat muscle minces. In two experiments with human muscle, failures were also recorded. However, with these preparations pyruvate utilization as well as oxygen uptake was small and the preparations rapidly lost their activity *in vitro*. It is therefore a matter of conjecture as to how formidable this failure is in its bearing on the problem.

Our general conclusion is that our experiments have, in the main, confirmed those of Rice and Evans, but we are not prepared as yet on the basis of our own or their published experiments to conclude categorically that insulin is concerned in some reaction which directly accelerates the utilization of pyruvate by muscle.

SUMMARY

1. Minces of pigeon breast muscle were subject to a fore period with and without insulin. Pyruvate was then added and its utilization determined during a final period. Significant increases of pyruvate utilization were observed in the insulin-treated preparations.

2. In similar experiments with muscle slices significant insulin effects on pyruvate utilization were also observed.

3. In contrast, coarse homogenates of muscle which vigorously oxidized pyruvate failed to be influenced by insulin.

4. Muscle minces subjected to an initial period with and without insulin and then equilibrated with added malonate, pyruvate, and malate or fumarate showed positive insulin effects on pyruvate utilizations in half of the cases. With oxalacetate in place of the malate or fumarate, no such effects were observed.

5. Rat muscle minces in similar experiments failed to show any insulin effects. In these cases the pyruvate utilization was very low.

6. Miscellaneous experiments on the effect of pH, insulin concentration, length of fore period, etc., were also performed.

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THE PREPARATION OF OPTICALLY ACTIVE ISOCITRIC ACID FROM BRYOPHYLLUM LEAF TISSUE

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d-Isocitric acid[1] has, in recent years, attracted much interest as one of the organic acids concerned in reactions catalyzed by the enzymes of various animal and plant tissues. A demand has accordingly arisen for specimens of this rare substance for use in investigations of the enzyme systems involved. The fortunate observation that *d*-isocitric acid is present in the leaves of succulent plants of the family Crassulaceae in rather large proportions (5–7) suggested that a procedure could be developed whereby suitable preparations might be readily obtained. Such a procedure is described in the present communication.

Preparation of Material—Two species, *Bryophyllum fedtschenkoi* and *B. calycinum*, were used exclusively for the present work but other closely allied species would probably serve as well. *B. calycinum*[2] is commonly available in the greenhouses of university departments of botany, since the leaves are often used for demonstrations of the unusual manner of propagation of this plant; *B. fedtschenkoi* is less frequently found but has some advantage for the present purpose over *B. calycinum*, inasmuch as propagation is a little easier and isocitric acid is obtained from it in higher yield and is somewhat more easily purified.

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* Died November 20, 1947.

[1] The nomenclature of natural optically active isocitric acid is somewhat confusing. Inasmuch as the free acid and its sodium salt are both *dextrorotatory* in water solution, the isomer present in plants must be designated *d*-isocitric acid or *d*-isocitrate. The lactone and its dimethyl ester are *levorotatory* in methyl alcohol solution and must therefore be designated *l*-isocitric lactone and *l*-dimethyl isocitrate lactone. However, the rotation of the free acid is usually determined in the presence of ammonium molybdate and the complex formed is very strongly *levorotatory;* accordingly there is a tendency to refer to this in naming the acid. Krebs and Eggleston (1), for example, designate it as (−)-isocitric acid. Discussions of the facts are to be found in several papers (2–6). It is probable that the configuration of the α-carbon atom of natural isocitric acid is the same as that of natural *l*-malic acid, which is the same as that of the L_s family to which the protein amino acids belong, but this has not yet been rigidly established. Pending the adoption for α-hydroxy acids of a more rational nomenclature founded upon configuration, there is no alternative to the careful use of the prefixes in their original meaning, *i.e.* to designate the actual direction of the rotation under defined conditions.

[2] Also known as *Bryophyllum pinnatum* and by the common name "life-plant."

To propagate the plants, well grown leaves with deep marginal indentations are picked and placed flat upon moist sand, being protected from light until roots have become established at the indentations (8 to 10 days). The leaves are then uncovered and the sand is kept moist with a suitable culture solution[3] until the plants are several centimeters high.[4] Application of the culture solution is necessary three or four times a week. The small plants are then separately transplanted into soil or sand in individual crocks. If sand is used, the same culture solution is applied regularly as needed. In the greenhouse, the plants will have grown sufficiently for use in 3 to 4 months; during the summer season they may be grown out of doors. For each preparation of approximately 10 gm. of isocitric acid, five to seven plants are required; the fresh leaves will weigh 3 to 4 kilos and will give 200 to 300 gm. of dry tissue.

Because of the wide diurnal variation in malic and citric acid content of the leaves of *Bryophyllum* plants (isocitric acid shares to only a moderate extent in the diurnal changes (9, 10)), it is necessary to harvest the leaves at a low point in the cycle so that the relative proportion of isocitric to the other acids shall be as high as possible. This situation occurs in the afternoon of sunny days. Accordingly, the leaves are stripped from the plants at from 3 to 5 p.m. on a bright sunny day and at once placed on trays in a drying oven at 80–90°; when crisp, the tissue is broken up and powdered in a Wiley mill. This powder may be stored indefinitely.

Extraction of Organic Acids—Organic acids may account for from 15 to 25 per cent or even more of the dry weight of *Bryophyllum* leaves, depending upon the species and the time of day the leaves are collected. In samples picked in the afternoon, the total organic acids are low but isocitric acid may make up as much as 70 per cent of the residual acidity. The other acids present are chiefly malic and citric, together with minor proportions of other substances. The following description refers to the preparation of isocitric acid in approximately 10 gm. units, a convenient quantity to deal with in laboratory scale apparatus. The figures given for weights of products are approximately those that will be obtained if *Bryophyllum calycinum* is used, this being the most commonly available species.

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[3] 0.5 M solutions of four salts are prepared and the culture solution is made by mixing 20 ml. of potassium dihydrogen phosphate, 100 ml. of calcium nitrate, 40 ml. of magnesium sulfate, and 100 ml. of potassium nitrate. To this are added 20 ml. of a solution of ferric tartrate (2.6 gm. per liter) and 20 ml. of a solution that contains 2.85 gm. of boric acid and 2.03 gm. of magnesium sulfate tetrahydrate per liter. The mixture is then diluted to 10 liters with tap water, and adjusted to pH 5.9 to 6.2 by the addition of 2 to 5 ml. of 1.0 N sulfuric acid as required (8).

[4] An alternative and somewhat superior method of propagation consists in placing the leaves on wire mesh trays in a dark cabinet where they are sprayed continuously with a fine mist of water. Rootlets begin to form in a few days and, when well developed, portions cut from the leaves are transferred to sand in flats and watered with culture solution. A higher yield of young plants is obtained in this way.

For the extraction, 200 gm. of dry leaf tissue are stirred to a uniform pulp with 300 ml. of 80 to 85 per cent alcohol, to which is added sufficient 18 N sulfuric acid to bring the mixture to pH 1.8 to 2.0 as measured in aqueous suspension. Usually, 30 to 50 ml. are required, the exact amount being ascertained by mixing 0.5 gm. of the dry powder with successive 0.5 ml. increments of 1.0 N sulfuric acid, diluting the mixture to 10 ml., and measuring the pH with a glass electrode. The suspension is diluted with 700 ml. of 80 per cent alcohol, 10 gm. of norit are added, and the mixture is heated on the steam bath for 15 minutes with frequent stirring. The tissue is filtered off, washed with dilute alcohol, and extracted twice more with 600 ml. of hot alcohol under the same conditions. The combined extracts are then cooled and filtered through paper pulp, again heated nearly to the boiling point and treated with aqueous barium hydroxide solution saturated at 70–80°, and added slowly with stirring, until definitely alkaline to phenolphthalein paper; about 1 liter will be required. After being allowed to digest on the steam bath for half an hour, the precipitate of barium salts of the organic acids is filtered hot on two 18 cm. Büchner funnels fitted with No. 4 Whatman filter paper covered with a thin layer of Celite. The precipitate is washed repeatedly with liberal amounts of hot 40 per cent alcohol.

Alternatively, basic lead acetate in 40 per cent aqueous solution may be used for the precipitation of the organic acids, the reagent being added until a filtered test sample gives no further precipitation. The lead salts are decomposed with sulfuric acid as described for the barium salts. However, it is necessary to filter off and wash the lead sulfate and concentrate the solution before removing the excess of sulfuric acid quantitatively with barium hydroxide.

The precipitate is made into a thick slurry in water in an enamel ware pail with the aid of a wire beater and transferred to a large beaker through a wire mesh screen, all lumps being broken up with a brush. The suspension, at a volume of from 3 to 4 liters, is heated on the steam bath and treated with 18 N sulfuric acid with continuous stirring until a *small* excess is present (30 to 50 ml. will be needed). After making certain that the reaction is complete, dilute barium hydroxide solution is added with stirring until tests on filtered or centrifuged 5 ml. samples are negative for both sulfuric acid and barium ion; 25 gm. of norit are added, and the solution is again heated for about 15 minutes with stirring and is allowed to settle overnight. The clear solution is then siphoned off, the barium sulfate is centrifuged and washed twice with liberal amounts of hot water, and the combined solutions are filtered through Whatman No. 4 paper and concentrated *in vacuo* to about 250 ml. For this, and for subsequent evaporations *in vacuo*, a special still head described some years ago (11) is desirable, since it permits the interchangeable use of short ring neck flasks of different sizes.

Dehydration of Isocitric Acid to Its Lactone—The clear solution is transferred to a 1 liter tared flask and concentrated *in vacuo* in a boiling water bath until no more water distils and for about half an hour thereafter. The vacuum is released and heating is continued for another half hour, when the vacuum is again applied and distillation renewed. Several alternate cycles of heating and distillation for about 15 minutes each are then carried through, after which the flask is cooled and weighed. The dark red viscous sirup should weigh close to 30 gm. and most of the isocitric acid is now present as the lactone.

Esterification of Isocitric Lactone—A volume of absolute methyl alcohol in ml. equal to 5 times the weight of the sirup in gm. is added together with slightly more than 1 volume of redistilled boron trifluoride etherate (b.p. 51–52° at 22 mm.), and the mixture is heated under a reflux condenser on the steam bath, occasionally being shaken. The sirup gradually dissolves and heating is continued for a total of about 5 hours. After being chilled and filtered if any separation of solid material occurs, most of the alcohol is distilled off and the residue is transferred with the aid of methyl alcohol to a 200 ml. flask which is connected to a vacuum fractionation apparatus equipped with a short column. The methyl alcohol is first removed; the excess boron trifluoride etherate then distils and is followed by the dihydrate at an oil bath temperature of 100–110° at 5 to 7 mm. Evidence of the presence of a small amount of dimethyl malate may then be seen. The temperature of the bath is gradually raised to 160–165° until distillation has practically ceased and is held there for about 15 minutes. This temperature must not be exceeded or dimethyl isocitrate lactone will distil and crystallize in the condenser. The distillate is discarded. This operation removes the catalyst and the water together with any dimethyl malate present. However, the greater part of the malic acid has been converted into complex products of dehydration which form oils and tars that do not distil under the conditions. Esters and dehydration products of citric acid also remain undistilled.

Isolation of Crude Dimethyl Isocitrate Lactone—The viscous black residue in the flask (about 31 gm.) is transferred with the aid of hot absolute ethyl alcohol to a beaker with a total volume of 150 ml. Norit equal to 10 per cent of the weight of the residue is added and the boiling solution is filtered in a steam-jacketed funnel through Whatman No. 4 paper moistened with water. Filtration is often slow and the heated funnel is necessary. The norit is washed with a total of 100 ml. of hot alcohol and the dark colored but clear filtrate is chilled overnight, being stirred occasionally. The buff-colored crude product which crystallizes is then filtered, washed with cold absolute alcohol, and dried at 70°; about 12 gm. (melting point in range from 99–105°) are obtained. An additional small but equally pure crop can be obtained by concentration of the mother liquor. The total yield should be about 13 gm., the equivalent of about 60 per cent of the isocitric acid present in the original alcohol extract of the tissue. The balance of the isocitric acid is present as compounds that cannot be brought to crystallize.[5]

Purification of Dimethyl Isocitrate Lactone—The crude product is ground to a fine powder and dissolved in 7.5 times its weight of hot water at a temperature that should not exceed 90°. After all but a little residual tar is in solution, one-fifth of its weight of norit is added together with a little Celite and the solution, after a short digestion period at 90°, is filtered on a steam-jacketed funnel, the residue being washed with hot water. After being chilled overnight, the colorless crystals are filtered, washed with ice water followed by cold alcohol, and are dried at 70° or in air at room temperature. The recovery is 77 to 80 per cent of material that melts between 104–106°. A small second crop that raises the yield to 83 to 85 per cent can be obtained from the mother liquor by evaporation to one-tenth its volume. If this crop has a low melting point, it is recrystallized from hot absolute alcohol. Different preparations gave specific rotations in methyl alcohol solution in the range from $-63°$ to $-65°$. After saponification, the specific rotation of the free acid in water in the presence of ammonium molybdate was $-362 \pm 4°$ as the mean of seven determinations on three pools of from one to ten preparations each, in agreement with an earlier figure of $-363°$ (5) obtained as the mean of observations on a small number of preparations. As further evidence of identity, 0.100 gm. each of a series of five preparations was saponified in the presence of excess 0.1 N sodium hydroxide by being heated on the steam bath for 15 minutes. Titration to phenolphthalein showed that from 14.80 to 14.85 ml. of alkali were neutralized. Multiplied by the factor 67.38, these figures show that the purity was in the range 99.7 to 100.1 per cent.

Isocitric Acid Lactone—Although the dimethyl ester of the lactone is the most desirable compound of isocitric acid for isolation purposes (2) and is also to be preferred as a form in which to preserve the substance, occasions may arise in which the lactone itself may be needed. To obtain this, 20 gm. of the ester are boiled with 200 ml. of 3 N hydrochloric acid under a reflux condenser for 6 hours. The lactone ring is opened by acid hydrolysis but closes again during the dehydration of the sirup. Hydrolysis is accompanied by slight decomposition. The solution is treated with 5 gm. of norit, filtered, and concentrated *in vacuo* to a sirup, heating being then continued until the sirup solidifies. The hard cake of crystals is broken up, transferred to a dish in a vacuum desiccator over drierite and a dish of solid sodium hydroxide. The desiccator is evacuated and placed in the oven at 105° for 2 hours and then at room temperature for an additional 48 hours, when all hydrochloric acid will have been absorbed. The crude lactone weighed 17.1 gm. (theory 17.3 gm.), sintered at 141°, and melted at 148°. Titration showed that 94 per cent of the material was present as lactone while, after saponification with alkali, 97 per cent was present as isocitric acid. Purification is effected by recrystallization from hot chloroform, the recovery being about 91 per cent. The purified lactone sintered at 148° and melted at 152–153°. It gave no depression of melting point when mixed with a highly purified specimen of melting point 153–154°. After hydrolysis with alkali, the specific rotation of the isocitric acid in the presence of ammonium molybdate was −366°.

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[5] When pure isocitric acid is carried through the same series of operations, the yields of dimethyl isocitrate lactone were from 80 to 90 per cent. The reasons for the lower yields secured when the mixture of acids in tissue extracts is esterified are being investigated; under these circumstances, reactions more complex than simple esterification seem to occur. The desirability of using tissue samples collected in the afternoon at the time of low malic acid content is obvious in the light of the behavior described.

The optically active lactone, in contrast with synthetic *dl*-isocitric lactone (12), is far too soluble in ethyl acetate for this solvent to be used for crystallization, even when toluene is added. The recovery from the mixed solvents used in equal volumes was only about 60 per cent.

Isocitric Acid—To prepare a solution of the sodium salt of isocitric acid for experimental purposes, the desired quantity of the methyl ester of the lactone is dissolved in water and a small excess over the calculated quantity of standard sodium hydroxide solution is added. The solution is heated on the steam bath until methyl alcohol is expelled and is neutralized to the desired pH. The lactone may be converted to the acid by a similar procedure.

DISCUSSION

The efficiency of the extraction of the acids from the dry leaf tissue by the procedure advocated may be judged from the data of Table I. Although less effective than extraction of the acidified tissue with ether, as in the analytical determination of total organic acids (13), hot alcohol is much to be preferred to water for practical work on the present scale, since such components as inorganic salts and pectins are not dissolved. Acidification is necessary to liberate the acids from their salts.

Table II shows the approximate composition of a number of lots of crude dimethyl isocitrate lactone, each analysis referring to a pool of several preparations. The individual preparations were made from quantities of dry tissue that ranged from 40 to 200 gm. each. The analyses, particularly those of the preparations from *Bryophyllum calycinum*, do not account for the whole of the material because of the presence of tar-like impurities. The proportion of dimethyl isocitrate lactone was calculated from obser-

TABLE I

Organic Acids of Bryophyllum Leaf Tissue

The figures refer to 200 gm. samples of tissue harvested in the afternoon of sunny days.

	B. fedtschenkoi			*B. calycinum*		
	Dry leaves	Alcohol extract		Dry leaves	Alcohol extract	
	gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
Total acids	54.71	47.06	86	36.46	32.07	88
Citric acid	4.61	4.29	93	2.73	2.63	96.5
Malic "	15.80	15.28	96.7	8.64	8.38	97
Isocitric acid (by difference)*	34.3	27.5	80	25.1	21.1	84

* Calculation by difference involves the assumption that no other acids are present. It has been shown for *B. calycinum* that the total quantity of other acids is in fact small (5).

TABLE II

Composition of Crude Dimethyl Isocitrate Lactone from Two Species of Bryophyllum Leaves

Species	No. of preparations in pool	Average yield of crude ester*	Limits of m.p. of individual preparations	Composition of pooled crude ester			Yield of pure ester†
				Dimethyl isocitrate lactone	Trimethyl citrate	Dimethyl malate	
		per cent	*°C.*	*per cent*	*per cent*	*per cent*	*per cent*
B. fedtschenkoi	4	70.1	100–105	89.9	1.31	6.29	52.6
	2	63.7	103–105	96.2	0.52	2.88	52.9
	5	63.6	100–104	89.4	0.66	5.27	50.9
B. calycinum	6	59.0	99–104	85	0.83	3.32	47.0
	6	54.6	101–105	83	1.33	3.58	45.0

* Yield calculated from isocitric acid content of dry tissue as obtained by difference.

† The various samples melted fairly sharply beween 104–106°; the specific rotations in methyl alcohol were from −63° to −65°, and the specific rotations in the presence of ammonium molybdate after saponification averaged −362 ± 4°.

vations of the rotation in the presence of ammonium molybdate after saponification, the influence of the small proportion of malic acid being neglected. Malic and citric esters were determined by the usual methods after saponification.

The final yields of purified dimethyl isocitrate lactone shown in the last column of Table II were computed from the isocitric acid content of the alcohol extracts. Inasmuch as this quantity was obtained by difference, no allowance being made for the presence of other organic acids, the figures shown are doubtless underestimates.

The chief problem in the preparation of isocitric acid from natural sources is the separation from citric acid which invariably accompanies it. Fractionation of the ethyl esters has been found to be extremely inefficient because of the similarity in boiling points (5); nor can the methyl esters secured after lactone formation and esterification with hydrogen chloride as catalyst be separated by fractional crystallization save at the expense of serious losses. Only from 15 to 30 per cent of the isocitric acid present could be isolated in this way as dimethyl isocitrate lactone, the balance of the acid being present in uncrystallizable oils that were found to be rich in trimethyl isocitrate. The lactone itself cannot be used for isolation because, unlike the synthetic material, the optically active natural substance does not crystallize well in the presence of impurities. However, the dimethyl ester (2) has excellent properties.

The use of boron trifluoride etherate as catalyst of the esterification of isocitric lactone solved the problem of obtaining moderately good yields of the ester, it being possible with this catalyst to esterify pure preparations of both the optically active and the synthetic lactone with yields approaching 90 per cent, although none whatever was secured from synthetic *dl*-lactone when hydrogen chloride was used. Nevertheless, boron trifluoride cannot be successfully used to catalyze the esterification of citric and malic acids. With citric acid, a yield of only 40 to 50 per cent of trimethyl citrate was obtained, while malic acid yielded only traces of ester. Furthermore, since dimethyl malate distils at about the same temperature as boron trifluoride dihydrate, separation is difficult. After such esterifications, the greater part of the malic acid was present as a viscous oil that did not distil at a bath temperature as high as 250° at 5 to 7 mm. pressure. Although the oil was acid in reaction, very little was soluble in water and only a small proportion of free acid groups was present. After saponification, about 76 per cent of malic acid was present in the oil together with 4 to 5 per cent of fumaric acid, which was isolated by fractional crystallization and identified by the melting point (179–180°) of the benzylthiouronium salt (14). Boron trifluoride clearly acts upon malic acid as a strong dehydrating reagent.

This observation explains why only traces of dimethyl malate are observed during the distillation of the boron compounds from the esters and accounts for the pigmented tars found as contaminants of the crude dimethyl isocitrate lactone. It also accounts for the presence of small quantities of fumaric acid frequently observed in the mother liquors of the crystallization of the lactone ester after saponification. Dimethyl fumarate sublimes at 100° and melts at 103–104°. If present as a contaminant of dimethyl isocitrate lactone, the melting point of this substance is greatly depressed. Accordingly the melting point of the crude isocitric acid derivative is an important criterion of purity and, together with the optical properties, serves as evidence of freedom from dimethyl fumarate. However, no case has yet been encountered in which dimethyl fumarate separated along with the isocitric acid derivative.

The present observations suggest that boron trifluoride etherate, although uniquely valuable for the isolation of isocitric acid, should not be used as a catalyst of esterification where the interest is centered upon the recovery of malic or citric acids.

SUMMARY

A method is described to prepare optically active *d*-isocitric acid from the dry leaf tissue of *Bryophyllum fedtschenkoi* or *Bryophyllum calycinum* collected in the afternoon of sunny days at the time of low malic acid content. The organic acids are extracted with hot diluted alcohol and precipitated as barium salts. After liberation from the salts, the aqueous solution of the acids is concentrated to a sirup which is heated to convert isocitric acid to its lactone. Esterification with methyl alcohol in the presence of boron trifluoride etherate as catalyst yields the levorotatory trimethyl isocitrate lactone which is isolated by crystallization from hot ethyl alcohol and purified by recrystallization from hot water. Approximately half of the isocitric acid calculated by difference to be present in the extract is obtained as pure dimethyl isocitrate lactone. The failure to obtain higher yields may be in part ascribable to complex dehydration reactions that occur during the esterification of the mixture of acids in the presence of boron trifluoride. Nevertheless, boron trifluoride is much to be preferred to hydrogen chloride for this special purpose.

Dimethyl isocitrate lactone is the most desirable derivative of *d*-isocitric acid both for isolation and for storage. It can be converted with little loss into the free lactone by hydrolysis with hydrochloric acid or into isocitrate without loss by saponification with a slight excess of alkali.

Observations are recorded on the complex reactions which occur when malic acid is esterified with boron trifluoride etherate as catalyst.

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THE CYTOCHROME *c*-AZIDE COMPLEX

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Since both cyanide and azide have been shown to block the oxidation of cytochrome *c* by cytochrome oxidase (1), it has been generally accepted that the toxicity of these substances is due to this property. A considerable amount of evidence has accumulated, however, indicating that azide and cyanide do not inhibit respiration in a parallel manner under all conditions. Thus Stannard (2) was able to demonstrate that only a fraction of the respiration of stimulated frog muscle is sensitive to azide and that the azide-insensitive fraction is identical with the respiration of the resting muscle. Both resting and activity respiration, on the other hand, are completely inhibited by cyanide.

Fischer, Henry, and Low (3) found similarly that the extra respiration resulting on fertilization of the sea-urchin egg is inhibited by azide, while the respiration of the unfertilized egg is not azide-sensitive. With respect to the action of cyanide on this system there is some confusion, some workers (4, 5) having reported that the respiration of the unfertilized egg is cyanide-insensitive. However, Robbie (6) attributes these findings to a failure to maintain the cyanide concentration in the manometric experiments and to a secondary oxygen uptake induced by the higher concentrations of cyanide. He presents evidence indicating that the respiration of both the unfertilized and fertilized egg is cyanide-sensitive.

To account for this fundamental difference in the effect of cyanide and azide on respiration, it has been postulated that an alternative pathway, independent of the cytochrome-cytochrome oxidase system, accounts for the azide-insensitive resting respiration (2, 5). Thus far, however, no evidence has been presented as to the nature of this pathway. Horecker and Kornberg (7) have demonstrated that ferricytochrome *c* combines with cyanide ion under physiological conditions, and it became of interest to study the action of azide on this substance, since with the isolated compound the quantitative as well as the qualitative aspects of the reactions could be examined.

EXPERIMENTAL

Cytochrome c—This was prepared from calf heart by the recently modified method of Keilin and Hartree (8). The Fe content of this preparation as determined by the method of Delory (9) was 0.33 per cent. The cytochrome *c* solutions were acidified to pH 4.0 with 0.1 N HCl and aerated to insure complete oxidation before neutralization and addition of buffer for each experiment.

Azide Solutions—Azide solutions were prepared from Eimer and Amend sodium azide and diluted in 0.05 M phosphate buffer unless otherwise indicated.

Succinic Dehydrogenase—This was prepared from rat heart by grinding with sand in 2 volumes of M/15 Na_2HPO_4 and centrifuging lightly.

Spectrophotometry—The spectrophotometric measurements were made with the Beckman spectrophotometer, with a spectral band width which varied from 7 A in the blue to 30 A in the red and infra-red. The concentration of the cytochrome *c* solutions was determined at 5500 A after reduction with $Na_2S_2O_4$ and calculated with the extinction coefficient of 2.80×10^4 (concentration in moles per liter). The values obtained were in agreement with those predicted from the Fe content. When concentrated solutions of sodium azide were prepared, these were found to have a slight yellow to red color and appropriate blank measurements were made to correct the absorption. All measurements were made in cells having a length of 10.0 mm.

Absorption Spectra

Azide was found to form a well defined complex with ferricytochrome *c* with a spectrum differing significantly from that of free cytochrome, as is shown in Figs. 1 and 2. The point of maximal absorption is shifted from 5300 to 5400 A, an even greater shift than is observed with the cyanide complex, and a small new band appears at about 5700 A. The band for ferricytochrome *c* at 6925 A[1] is absent in the azide complex, as it is in the cyanide complex, but below 6500 A the absorption is increased. The extinction coefficients for the azide complex were calculated from data obtained with solutions containing 0.16 and 0.67 M sodium azide, for reasons indicated in the following section.

Dissociation Constant

Two wave-lengths, 6950 and 6300 A, at which the azide complex differs greatly in absorption from the free cytochrome *c*, were used for the determination of the dissociation constant. Fig. 3 shows the effect of increasing concentration of azide on the calculated extinction coefficients. At 6950 A

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[1] In a recent review Theorell (10) has confused this band with some previously reported by Bigwood, Thomas, and Wolfers (11). These authors described bands at 6400 to 6450 A and at 6750 A, which were obtained with ferricytochrome *c* only in alkaline solution and which are evidently distinct from the band at 6925 A.

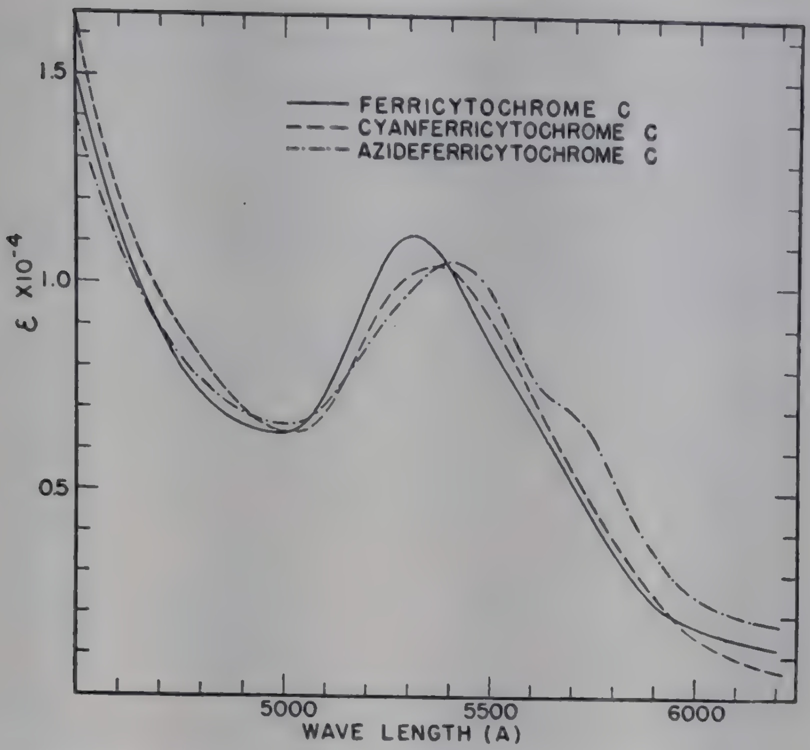

FIG. 1. Absorption spectra of cytochrome *c* and derivatives. Concentration, 3.2×10^{-5} M; cell length, 10.0 mm.

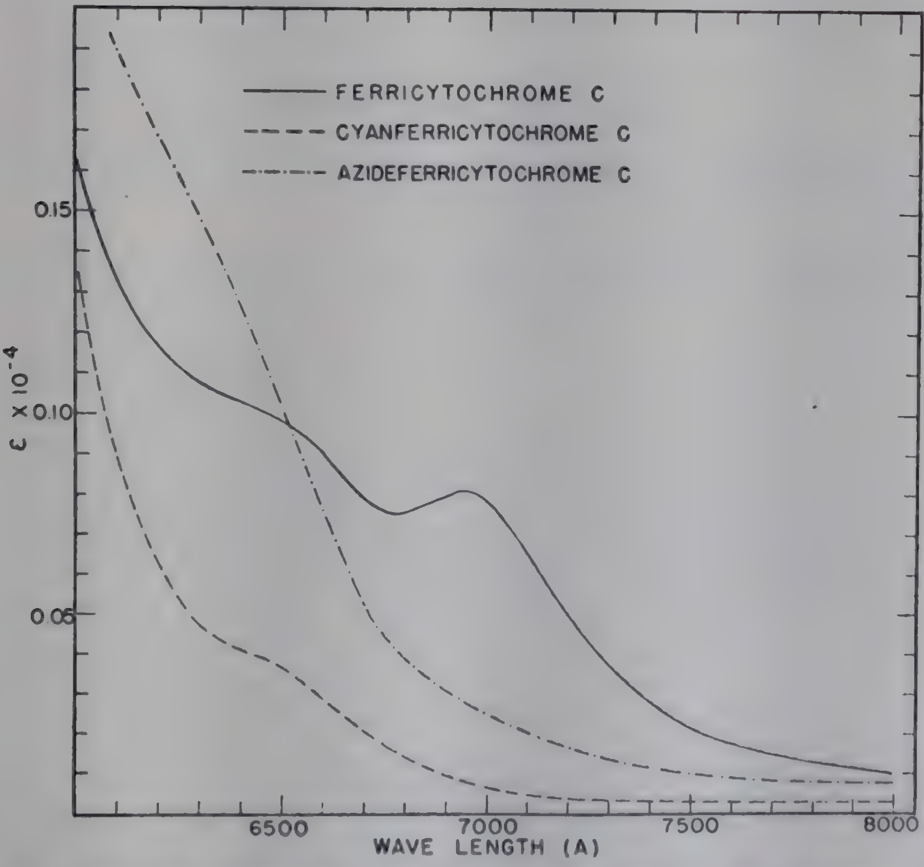

FIG. 2. Absorption spectra of cytochrome *c* and derivatives in the red region. Concentration, 3.2×10^{-4} M; cell length, 10.0 mm.

A there is a progressive decrease in absorption and at 6300 A a progressive increase, in both cases approaching asymptotic values which represent the extinction coefficients of the azide complex. However, in the very concentrated azide solutions required for complete formation of the complex, secondary effects, such as the formation of turbidity, interfered with the accurate determination of the extinction coefficients. These were obtained by a graphical method.

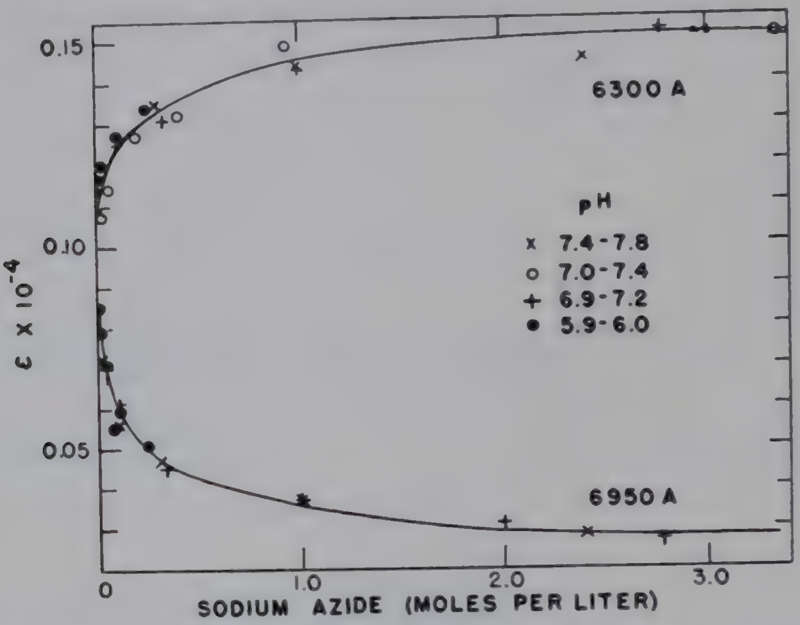

FIG. 3. Effect of azide on apparent extinction coefficients of ferricytochrome *c*. All solutions were diluted in 0.05 M phosphate buffer.

The rectangular hyperbolae shown in Fig. 3 may be represented by Equation 1,

$$(1) \qquad \epsilon_{\text{complex}} - \epsilon_{\text{observed}} = (\epsilon_{\text{complex}} - \epsilon_{\text{free}}) \cdot \frac{(N_3^-)}{K + (N_3^-)}$$

where $\epsilon_{\text{complex}}$, ϵ_{free}, and $\epsilon_{\text{observed}}$ are the extinction coefficients for the complex, the free ferricytochrome *c*, and the mixtures, respectively, (N_3^-) is the concentration of azide ion, and *K* the dissociation constant. From the reciprocal of Equation 1 a linear relation is obtained,

$$(2) \qquad \frac{1}{\epsilon_{\text{complex}} - \epsilon_{\text{observed}}} = \frac{K}{\epsilon_{\text{complex}} - \epsilon_{\text{free}}} \cdot \frac{1}{(N_3^-)} + \frac{1}{\epsilon_{\text{complex}} - \epsilon_{\text{free}}}$$

as is shown in Fig. 4. $\Delta\epsilon$ denotes the change in extinction coefficient produced by addition of azide. From the intercept the extinction coefficient of the complex may be calculated and from the slope the value of the dissociation constant. At the two wave-lengths, values for the dissociation constant of 0.16 and 0.14 were obtained.

In Fig. 5 the experimental points are fitted to the theoretical curve for a dissociation constant of 0.15, with 1 mole of azide ion assumed to combine with 1 of cytochrome *c*. Also shown are values calculated on the basis of the hydrazoic acid (HN_3) concentration calculated from the hydrolysis of the azide ion, with a value for the dissociation constant of HN_3 of 1.9×10^{-5} (12). In the pH range from 5.9 to 7.8 the points calculated on the basis of azide ion are in agreement with the theoretical curve, while on the basis of hydrazoic acid the points at each pH form a distinct set. It is thus concluded that, as in the case of cyanide, ferricytochrome *c* combines with 1 mole of azide ion.

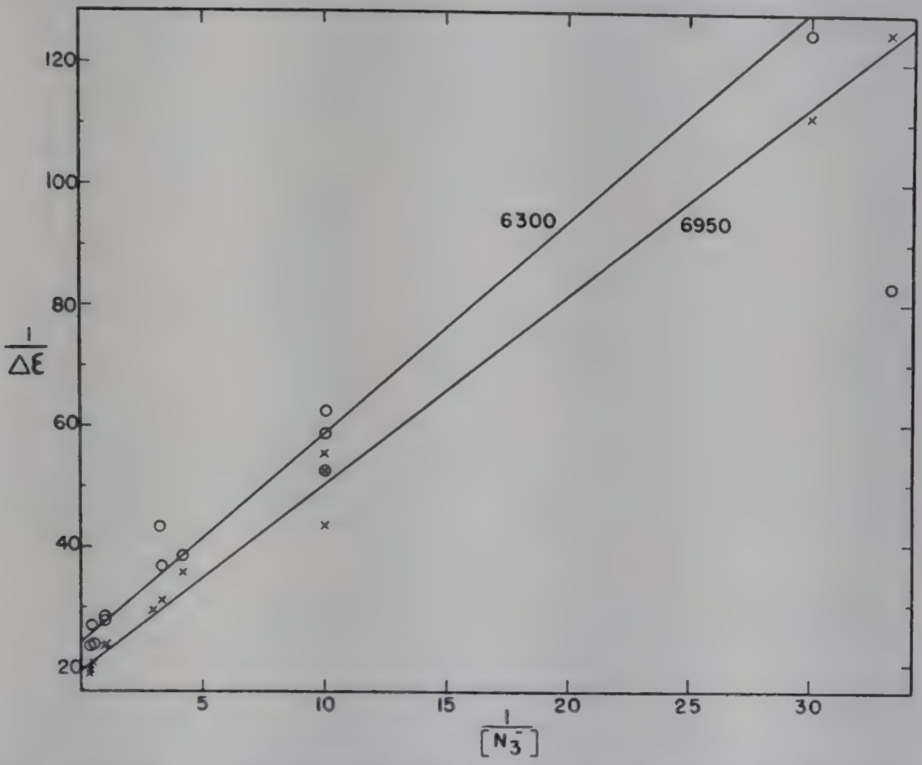

FIG. 4. Graphical determination of the extinction coefficients of azide-ferricytochrome *c* and the dissociation constant.

Reversibility

In order to establish that a true reversible equilibrium is reached when azide reacts with cytochrome *c*, the effect of succinic dehydrogenase on the complex was studied. At a concentration of N_3^- of 0.1 M, all of the cytochrome *c* is completely reduced in a few minutes, despite the fact that 40 per cent of the oxidized cytochrome is combined with azide. At somewhat higher azide concentrations the same general picture is observed.

although at concentrations high enough to bring about complete formation of complex the dehydrogenase is inhibited as well, so that no reduction is observed. This inhibition of succinic dehydrogenase is apparently similar to that observed by Potter (13) for chloride ion, and is obtained with sodium chloride and sodium nitrate as well as with sodium azide.

Effect of Azide on Autoxidation of Ferrocytochrome c

Although azide did not produce any changes in the absorption spectrum of ferrocytochrome *c*, it was found to accelerate the autoxidation of this substance markedly, as is shown in Table I. This effect on the rate of autoxidation was suggestive of the formation of an azide-ferrocytochrome *c* complex, with a decreased oxidation-reduction potential, but was subsequently concluded to be due to the catalytic action of an iron-azide complex formed from traces of inorganic iron. From Table I, it will be seen that added iron has a promoting effect on the azide catalysis, while iron in the absence of azide is without effect.

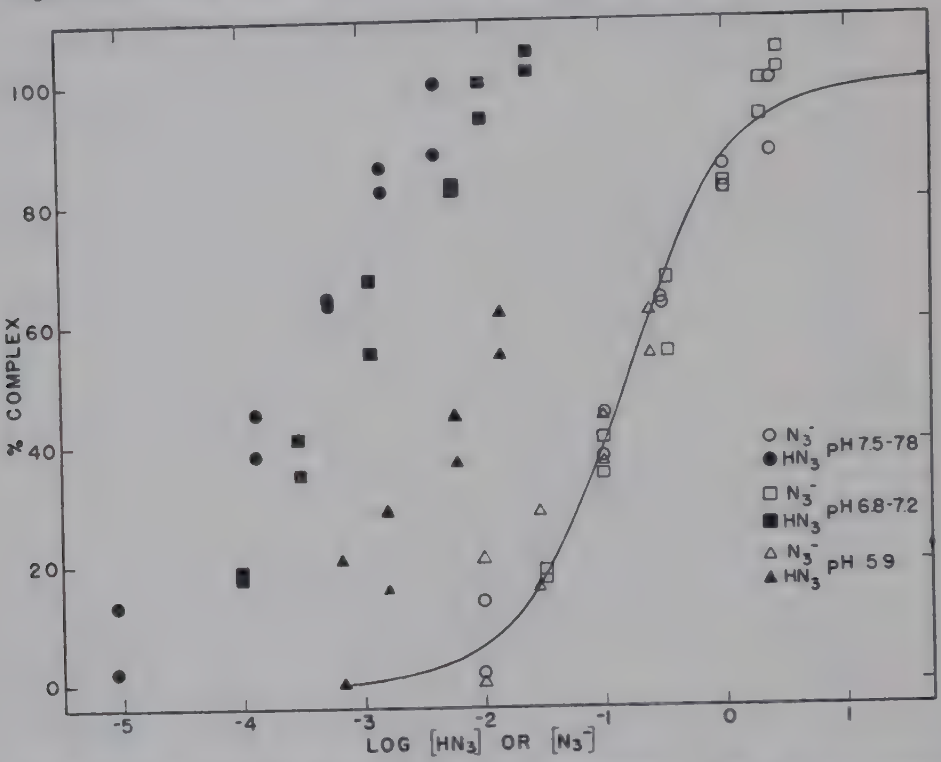

FIG. 5. Dissociation curve for azide-ferricytochrome *c*. The line represents the theoretical curve for a dissociation constant of 0.15, with 1 mole of azide assumed to combine with 1 mole of ferricytochrome *c*.

The ferric-azide complex is responsible for the color of concentrated azide solutions previously mentioned, and exhibits an absorption maximum at 4250 A. On reduction the color and absorption band disappear. From the change in absorption at 4250 and 5500 A, it was established that there is an instantaneous and quantitative reaction between the ferric-azide complex and ferrocytochrome *c*, following which, in the presence of air, the iron-azide complex is reoxidized; the rate of this step determines the over-all rate of ferrocytochrome *c* oxidation. Thus the alternate reduction and oxidation of the iron-azide complex brings about the complete oxidation of ferrocytochrome *c*, even though the iron is present in only minute quantities.

TABLE I

Effect of Sodium Azide and Iron on Autoxidation of Cytochrome c

Experiment No.	NaN_3	Fe	Rate of autoxidation
	mole per l.	*γ per cc.*	$k^* \times 10^4$
1	0	0	5
	0.03	0	9
	0.33	0	90
	1.00	0	890
2	0	0	20
	0.10	0	127
	0.10	1.33	1000
3	0	0	11
	0	1.33	15

* First order reaction velocity constant as defined in the succeeding paper.

TABLE II

Comparison of Azide and Cyanide Ferricytochrome c Complexes

	Azide	Cyanide
Dissociation constants at 25°, *mole per l.*	0.15	2×10^{-6}
% ferricytochrome *c* as complex at equilibrium at 25°, pH 7.4 in 0.001 M KCN or NaN_3	0.6	87
Rate of formation	Fast	Slow
pH effect	Absent	Present

DISCUSSION

In Table II the properties of the azide complex are compared with those of the cyanide complex as previously reported (7). While cyanide forms a stable complex with a dissociation constant of 2×10^{-6}, the rate of formation is slow; only half the cytochrome *c* is combined after 60 minutes in 0.001 M KCN at 25°, pH 7.4. On the other hand, the azide complex is much less stable, with a dissociation constant of about 0.15, but equilibrium is reached within the 30 seconds required for the spectrophotometer reading.

It is interesting to note the fraction of the ferricytochrome *c* which is combined at pH 7.4 in 0.001 M inhibitor at 25°, since these conditions are frequently used in studies *in vivo*. While formation of the cyanide complex at equilibrium is almost complete, 87 per cent, practically none of the ferricytochrome *c* would combine with azide.

The effect of pH on the degree of complex formation in azide and in cyanide is to be compared with the pH effect observed on the action of these inhibitors *in vivo*. The pH effect is absent in the range from 5 to 8 in the case of azide and present in the case of cyanide, indicating again that it is the ionic form of these substances which combines with the cytochrome *c*. Thus in the case of azide the concentration of the ionic form does not change appreciably with pH, since even at the acid end of the range practically all of the salt is ionized. In the case of cyanide, however, hydrolysis in the pH range studied is practically complete; in this case variations in pH produce large changes in the concentration of cyanide ion. These observations are not in accord with the pH effect observed *in vivo*, as is discussed in the succeeding paper.

The effect of azide on the oxidation of ferrocytochrome *c* suggests the possibility of a similar catalysis occurring in studies of the azide inhibition of respiration. To what extent this catalytic mechanism is able to replace the cytochrome oxidase system remains to be determined, but it should be considered whenever the inhibition of respiration by azide is incomplete.

It has been established that the affinity of cytochrome *c* for azide is very much less than for cyanide. Thus an alternative pathway of respiration might involve an iron-containing catalyst with similar affinities. In order for cytochrome *c* itself to account for the differential effects of azide and cyanide, it becomes necessary to introduce a cyanide- and azide-insensitive mechanism for the oxidation of ferrocytochrome *c*. The shift from resting to activity respiration would then involve an activation of the powerful cytochrome oxidase system, as has previously been suggested (2, 5). The activity respiration would thus be sensitive to both cyanide and azide, while only cyanide by combining with ferricytochrome *c* would affect the resting respiration. Experimental verification of this hypothesis must await the demonstration of an oxidative enzyme with the required characteristics.

SUMMARY

1. A complex is formed between ferricytochrome *c* and azide ion which contains 1 mole of azide ion per mole of cytochrome *c*. The complex is rapidly formed, but much less stable than the cyanide complex.

2. The absorption maximum is shifted from 5300 to 5400 A on formation of the complex and marked changes in absorption are observed in the red region.

3. The dissociation constant as determined spectrophotometrically is 0.15 at 25°. The formation of the complex is reversible.

4. The properties of the azide complex are compared with those of the cyanide complex and the possible physiological significance is discussed.

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THE IN VITRO INHIBITION OF CYTOCHROME OXIDASE BY AZIDE AND CYANIDE

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Differences in the action of the commonly employed respiratory inhibitors, cyanide and azide, have been described in studies on a number of tissues and microorganisms; *e.g.*, resting and active frog muscle (1, 2), mammalian salivary glands (3), embryonic fish hearts (4), and yeast (5). More recently differences in the action of these substances on the oxidation of various substrates by cell-free tissue preparations have been noted (6). Many of these findings and their interpretation were reviewed by Stotz (7) and Goddard (8) and specific examples were outlined in the preceding paper (9).

As a possible mechanism to account for these differences in the action of cyanide and azide on respiration, it has been postulated that a different pathway of metabolism, independent of the cytochrome-cytochrome oxidase system, may account for the azide-insensitive, cyanide-sensitive respiration. As discussed in the preceding paper, the possible nature of this pathway is still unclear, although it is not difficult to find examples of systems with these characteristics (6, 10).

As an alternative to the separate pathway hypothesis, Ball (11) has proposed that the oxidation-reduction potentials of the azide and cyanide complexes formed with cytochrome oxidase may be sufficiently different to account for the results. Specifically it was postulated that azide might combine with both the ferro and ferri forms of the enzyme, thus stabilizing the potential at a level sufficiently above that of cytochrome *c* to permit oxidation of the cytochromes at a diminished rate. In the presence of cyanide, on the other hand, the potential of the cytochrome oxidase system would be too low to allow any oxidation. Since cytochrome oxidase has not yet been isolated, direct measurement of the oxidation-reduction potentials is impossible and no experimental verification of Ball's suggestion is possible. However, it is clear that more fundamental study of the mode of action of these inhibitors, especially on cell-free preparations, is required. The present study was initiated with this general objective in view, and specifically to observe the effects of azide and cyanide on the oxidation of ferrocytochrome *c* measured spectrophotometrically.

EXPERIMENTAL

Enzyme Preparations—The majority of the experiments were carried out with a cytochrome oxidase preparation of rat heart, similar to that of Stotz and Hastings (12), except that the washings preceding the extraction were omitted. This preparation was found to be free of intact cells and could be pipetted readily. It was usable with some loss of activity for about 2 weeks if kept refrigerated. This was diluted 1:100 to 1:5000 in appropriate buffer, the dilution needed being determined by the pH and, to a lesser extent, the age of the preparation.

Rat brain and frog skeletal muscle were prepared as 2 per cent homogenates in distilled water with a Waring blendor, according to the technique described by Albaum, Tepperman, and Bodansky (13).

All enzyme preparations were kept iced at all times until pipetted into the absorption cells.

Reagents—The buffers used were 0.05 M phosphate (Sørensen), McIlvaine type, or 0.1 M borate (pH 9 to 9.1). The nature of the buffer altered somewhat the absolute rates of reaction at constant pH (the rates in phosphate buffer were higher than in McIlvaine's buffer at neutrality but lower at pH values below 6.5). However, the effects of azide and cyanide were independent of the nature of the buffer used.

Cyanide solutions were prepared fresh for each experiment from Baker's reagent grade KCN by use of volumetric flasks arranged to have only a negligible gas space when brought to volume. Stock solutions were made up in 0.1 N NaOH and dilutions from these prepared and adjusted to the desired pH just before the test. This procedure was found to minimize loss of HCN from the very dilute solutions employed in the final stage.

Sodium azide solutions were freshly prepared each day in the appropriate buffer.

Cytochrome *c* was prepared as described in the previous paper and estimation of its concentration and purity carried out in the same manner. Before use in the spectrophotometric test system a dilute solution was deoxygenated with previously purified nitrogen, reduced with a minimal quantity of $Na_2S_2O_4$, and any excess hydrosulfite removed by aeration. To check the possible formation of H_2O_2 from the small excess of hydrosulfite added in this procedure, preliminary tests were made in which the cytochrome was only partially reduced by $Na_2S_2O_4$. No change in results could be detected.

Spectrophotometry—The Beckman spectrophotometer was employed and density readings made at 5500 A as a function of time. All determinations with inhibitor present were paralleled by a control set prepared from the diluted enzyme at the same time. The oxidation of ferrocytochrome *c* was first order with respect to this substance, and the results are expressed as the first order reaction velocity constants. On the basis of fundamental equations already described by Altschul, Abrams, and Hogness (14), the concentration of ferrocytochrome *c* in moles per liter at any given time was calculated from the relation

$$C\text{ (ferrocytochrome } c) = \frac{d_t - \dfrac{\epsilon_{\text{ferri}}}{\epsilon_{\text{ferro}}} \cdot d_{\text{Na}_2\text{S}_2\text{O}_4}}{(\epsilon_{\text{ferro}} - \epsilon_{\text{ferri}})l}$$

where C (ferrocytochrome *c*) is expressed in moles per liter, ϵ_{ferro} and ϵ_{ferri} represent the extinction coefficients of ferro- and ferricytochrome *c* respectively, $d_{\text{Na}_2\text{S}_2\text{O}_4}$ is the optical density after addition of a slight excess of hydrosulfite, and d_t the optical density at any time, t. This becomes for a length, l, of 1.0 cm. and ϵ_{ferro} and ϵ_{ferri} equal to 2.80×10^4 and 0.84×10^4, respectively, at 5500 A

$$C\text{ (ferrocytochrome } c) = \frac{d_t - 0.3d_{\text{Na}_2\text{S}_2\text{O}_4}}{1.96 \times 10^4}$$

Correction for autoxidation of ferrocytochrome *c* was not necessary in most experiments, since this rate was negligible in comparison with the enzymatic rate. However, such determinations were included under each set of experimental conditions. It was noted that cyanide completely inhibited autoxidation, while azide at higher concentrations had a tendency to accelerate autoxidation (9).

Manometric Experiments—These were carried out in a conventional Barcroft-Warburg apparatus with reaction vessels of about 7 ml. capacity. The thin hind limb muscles of the frog were carefully dissected the evening before the experiment and treated as described by Stannard (1).

Results

Fig. 1 illustrates the proportionality of reaction rate to concentration of enzyme with the rat heart preparation. The rates are strictly proportional except for the point at 15×10^{-5} ml. of enzyme, which presented a much more rapid rate of oxidation than was employed in the inhibitor experiments, and which was too rapid for reliable measurement. It is concluded that the rates are sufficiently proportional to enzyme concentration to permit quantitative analysis of the inhibitor experiments.

Effect of Azide—Fig. 2 presents a composite plot representing the per cent cytochrome oxidase activity remaining as a function of the concentration of N_3^- and of undissociated acid, HN_3, at a series of pH values. Fig. 2 contains points obtained for the rat heart preparation only, but identical results were obtained at pH 7.4 with a homogenate of frog skeletal muscle.

A notable feature of the results plotted in Fig. 2 is the progression of apparent effect with pH if N_3^- ion concentration is used as the basis for

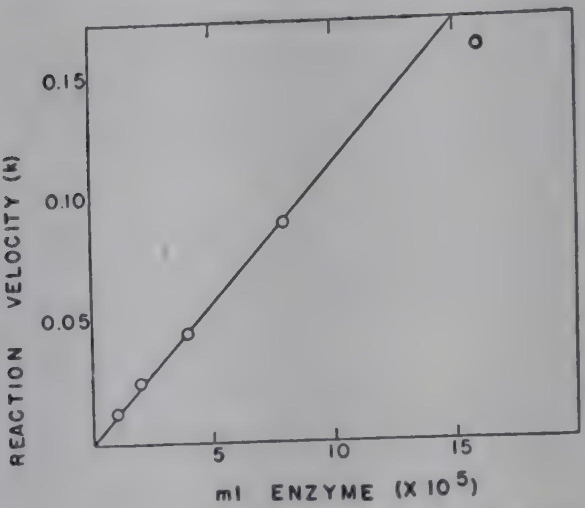

Fig. 1. Oxidation of ferrocytochrome *c* as a function of enzyme concentration (referred to the original enzyme preparation); pH 7.4, temperature 25°.

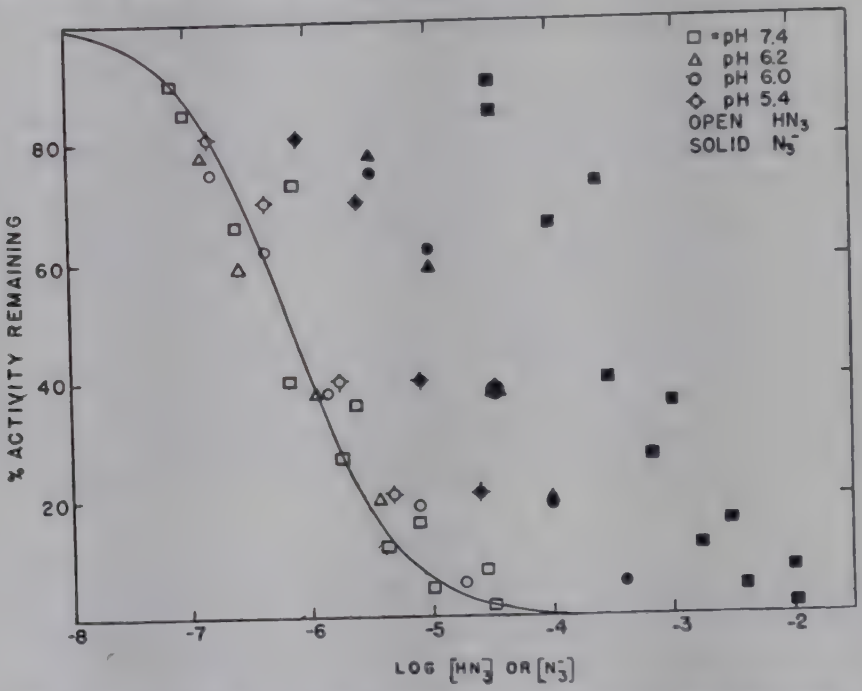

Fig. 2. The effect of azide on cytochrome oxidase activity as measured by oxidation of ferrocytochrome *c* in a rat heart preparation. The solid line is calculated from the mass law as described in the text, assuming a dissociation constant of 7×10^{-7}; temperature 25–26°.

comparison. Thus the solid symbols fit no single curve but would form a family of curves progressing to the left with decreasing pH. As a result there is a nearly hundred-fold difference in the N_3^- ion (and NaN_3)[1] concentrations required to produce a given effect as the pH progresses from 7.4 to 5.4. Actually this only reflects quantitatively the change in free acid concentration and suggests strongly that inhibition is proportional to the HN_3 concentration. This is completely borne out by the good fit to theory (Fig. 2) obtained when computation is based upon the HN_3 concentration. For these reasons, the dissociation constant and other characteristics are reported on the basis of a reaction between the free acid (HN_3) and the enzyme. As is indicated in the legend, the curve fitted to the open symbols is a theoretical one derived from the mass law and represents the expression

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[1] Since the free acid is always a relatively small fraction of the total azide in the pH range employed (0.17 at pH 5.4, 0.002 at pH 7.4), the relationship of effect to total NaN_3 and to N_3^- is very similar.

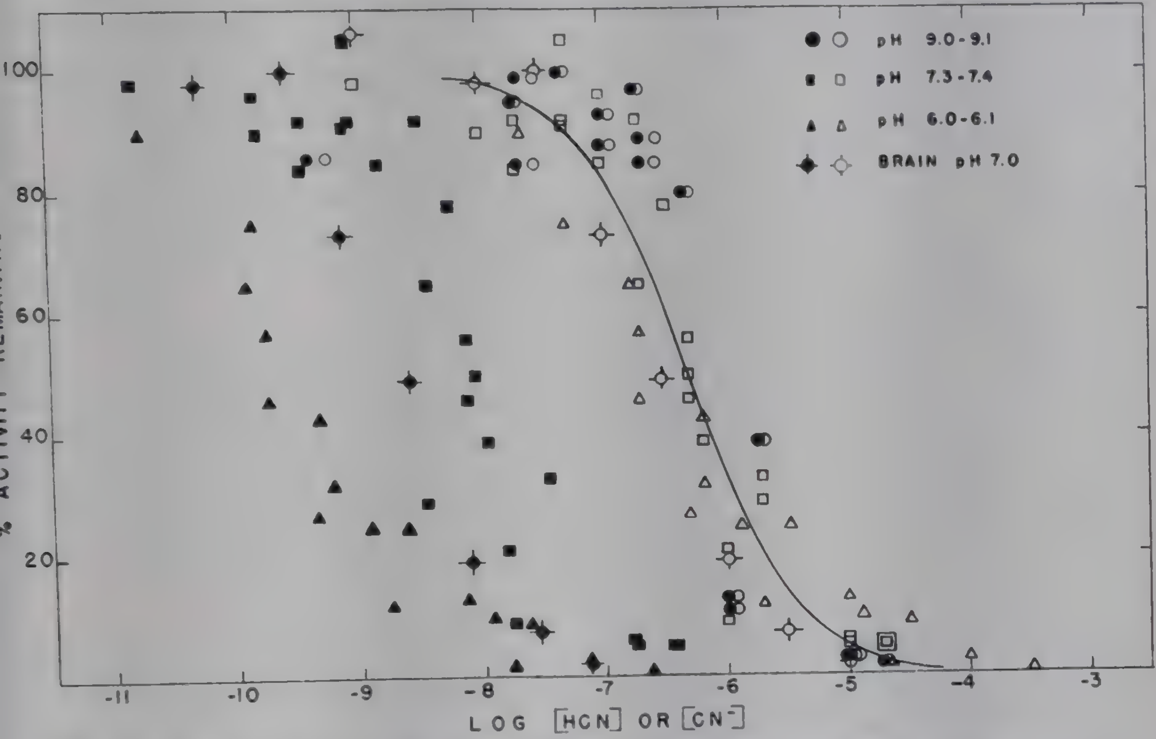

FIG. 3. The effect of cyanide on cytochrome oxidase activity of rat heart and brain preparations. The open symbols represent HCN concentrations, the solid symbols CN^- concentrations. The solid line is derived from the mass law as described in the text, assuming K for the enzyme-HCN complex $= 5 \times 10^{-7}$; temperature 25–26°.

$$\frac{\text{(Free enzyme)}}{\text{(Combined enzyme)}} \cdot (\text{HN}_3)^n = K$$

where (free enzyme) is the fraction of activity remaining (combined enzyme), the fraction of activity inhibited, n the number of molecules of azide combining with each enzyme molecule (or active group), and K the dissociation constant of the enzyme-azide complex. In these calculations the dissociation constant of HN_3 was taken as 1.9×10^{-5} (15), $n = 1$, $K = 7 \times 10^{-7}$.

Effect of Cyanide—Fig. 3 presents a composite graph for experiments in which the influence of cyanide on the enzymatic oxidation of ferrocytochrome *c* was determined at several H^+ ion concentrations. Rat heart and brain homogenates were employed as sources of the enzyme. The activity remaining is plotted as a function of log (CN^-) or log (HCN).

In spite of the precautions outlined in the experimental section for handling KCN solutions, the variability observed in these experiments was somewhat greater than in those with azide. Hence a larger number of tests was made. It is clear, however, that the effect of cyanide on both heart and brain preparations closely parallels that described above for azide. The points based on cyanide ion form a family of curves as a function of pH, while those relating effect to free HCN concentration fit reasonably well a theoretical curve derived from the mass law in the manner described above for azide. The dissociation constant for the enzyme-HCN complex is 5×10^{-7} and $n = 1$.[2] Thus the similarity in the action of azide and cyanide is marked and extends even to almost identical dissociation constants for the respective enzyme-inhibitor complexes.

Since the dissociation constant of HCN is very small (7.2×10^{-10} at 25° (15)), most of the cyanide is present as HCN except at the most alkaline pH. As a result there is very little difference in the inhibitory action of a given total KCN concentration at various pH values in the physiological range, a superficial contrast with the marked pH dependency seen with NaN_3. This same contrast has been observed in intact tissue studies (4). The basis for the phenomenon *in vitro* at least is now obvious.

Incidental to the above, it is also clear that, at pH 7.4 for example, cyanide will be a more effective inhibitor than azide on the basis of total salt at submaximal levels of inhibition. For example, at 10^{-3} M total salt and pH 7.4 the oxidase is completely bound to cyanide, but only 75 per cent is bound to azide. However, this is of importance only in determining the concentration range for exploration in experiments with intact tissues and does not represent a fundamental difference in action.

Manometric Experiments—A dependency of effect on pH was noted in Keilin's original observations on the effect of azide on yeast respiration (16), and, as noted above, both the presence of pH dependency in the case of azide and its absence in the case of cyanide were noted by Armstrong and Fisher (4). On the other hand, experiments with frog muscle (1) showed that the resting respiration remained insensitive to azide even when the pH of the medium was lowered to 4.6. In these earlier experiments, the respiration of stimulated muscle was studied at a single pH. In view of the hypothesis that cytochrome oxidase participates only in the respiration of stimulated muscle, a check of the effect of azide on the activity respiration at various pH values was considered of interest for comparison with the results *in vitro* already outlined.

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[2] The scatter of points in the cyanide tests necessitated trials of lines calculated on the basis of $n = 2$ and $n = \frac{1}{2}$, but the fit of these latter was obviously poorer than with $n = 1$.

Accordingly, the effect of selected NaN_3 concentrations on the oxygen uptake of frog muscle stimulated by immersion in 0.035 per cent caffeine (*cf.* (1)) was determined as a function of pH of the medium over the range 7.4 to 5.4. The results are summarized in Table I.

It is clear that there is a marked pH dependency in the effect of azide on the respiration of caffeinized frog muscle. Also it will be noted that the inhibition is not complete even at the lowest pH, since total respiration, not the increment due to caffeine, is reported in Table I. Since the intracellular H^+ concentration could not be determined, no attempt was made to place these data on the same basis as the spectrophotometric determinations. Also, penetration phenomena may modify the results obtained in experiments with intact tissues. However, the basic phenomenon is clearly present, and bears a close similarity to that observed in the oxidation of ferrocytochrome *c* measured spectrophotometrically.

TABLE I

Inhibition of O_2 Uptake of Caffeinized Frog Muscle by Azide at Various pH Values

Initial pH of medium	NaN_3	HN_3	Oxygen uptake (Q_{O_2})*			Per cent change	
			Control†	1st 30 min.	2nd 30 min.	1st 30 min.	2nd 30 min.
	$M \times 10^4$	$M \times 10^4$					
7.4	8.4	0.02	1.90	2.20	2.54	+16	+34
	8.2	0.02	1.56	1.80	2.16	+15	+39
7.1	8.9	0.04	4.57	4.16	3.72	−9	−18
	9.0	0.04	2.12	2.00	3.10	−6	+46
	9.0	0.04	2.56	2.62	3.08	+8	+20
	28.0	0.12	2.60	2.58	1.38	−1	−47
6.0	0.3	0.02	4.10	3.48	3.46	−15	−16
	9.1	0.45	5.76	1.78	1.18	−69	−80
5.6	8.2	0.94	2.74	1.18	0.76	−57	−72
	8.5	0.98	2.96	1.10	0.74	−63	−75
5.4	9.5	1.61	4.84	1.46	0.72	−70	−85
	9.5	1.61	6.98	2.26	1.44	−69	−79

* In c.mm. per mg. of wet weight per hour.

† Before addition of azide.

DISCUSSION

The experiments presented above demonstrate that the enzymatic oxidation of ferrocytochrome *c* can be completely inhibited by both azide and cyanide when a spectrophotometric test system and cell-free homogenates are employed. In addition, the properties of the reaction between enzyme and inhibitor are very similar with these two substances. There is no "azide-stable" fraction or other difference between azide and cyanide comparable to those seen in intact tissues (1–6) or to be expected from Ball's postulated behavior of the oxidation-reduction potentials of the enzyme-inhibitor complex (11). Unless the azide complex of cytochrome oxidase behaves differently *in vivo*, it is unable to oxidize cytochrome *c* and, therefore, could not account for the residual respiration obtained in the presence of azide. Thus other mechanisms, possibly still involving cytochrome *c*, but not the oxidase, must be sought for an explanation of the azide-insensitive, cyanide-sensitive respiration of intact tissues. The possibility that the inhibitor itself brings about qualitative changes in metabolism cannot be ignored (17), however, and further studies of this aspect with purified systems are in progress.

The effect of pH noted with azide in yeast and other intact cells and described above in muscle has frequently been attributed to permeability phenomena because of the more ready cellular penetration, in general, of uncharged particles. The appearance of this same phenomenon in cell-free preparations indicates that the explanation in intact tissues may be, in part at least, characteristic of the combination between enzyme and inhibitor. A study of the relative rôles of these two phenomena in intact cells is indicated, especially since it has been shown that certain local anesthetic bases enter *Arbacia* eggs as undissociated molecules but act in cationic form (18), while many barbituric acid derivatives both penetrate and act as undissociated molecules (19).

The experiments with cyanide were technically closely similar to those reported by Albaum, Tepperman, and Bodansky (13). Yet our results indicate a close agreement with equations derived from the mass law with *1* molecule of cyanide combining with 1 of enzyme, while Albaum *et al.* obtained a curve relating activity to log cyanide concentration, from which it would appear that 1 cyanide inactivates *4* enzyme molecules (or active groups). Consequently, their experiment with rat brain was duplicated exactly as described except for use of double the quantity of enzyme and slightly lower pH, and the results shown in Fig. 3 were obtained. Therefore, we are at a loss to account for the difference between these two series of experiments.

Except for the examples cited at the outset and above, it has not been possible to locate clear cases in the literature in which the reaction of an enzyme with cyanide or azide takes place with the free acid. Usually the form of combination is ignored, although many of the earlier studies on cyanide utilize terminology indicating that HCN was considered the active agent (*e.g.*, inhibition of *Atmungsferment* (20), catalase inhibition

(21), and peroxidase inhibiton (22)), but without formal proof or data which could be used for the necessary computations.

In the preceding paper (9), and in earlier work on the cyanide-cytochrome *c* complex (23), the reaction of azide and cyanide with ferricytochrome *c* was shown to involve the ionic forms, N_3^- and CN^-. Since the reaction with cytochrome oxidase involves the undissociated acid as shown here, the form of combination with ferrihemoglobin and ferrimyoglobin was considered of interest. The magnetic moment studies of Coryell, Stitt, and Pauling (24) show that the reaction between ferrihemoglobin and cyanide involves the CN^- ion. We have confirmed this finding, using a spectrophotometric method, and have extended the observations to include azide and ferrimyoglobin.[3] It was clear that the N_3^- and CN^- are the forms which combine with ferrihemoglobin and ferrimyoglobin. Thus the enzyme cytochrome oxidase appears in a somewhat anomalous position with respect to hemin type compounds of established structure. Interesting questions are raised, thereby, as to the nature of the combining groups in the enzyme and the structure of the oxidase as compared with well established hemin compounds.

SUMMARY

1. The oxidation of ferrocytochrome *c* by cytochrome oxidase preparations from rat heart and brain and from frog skeletal muscle was followed spectrophotometrically, and the effects of azide and cyanide investigated.

2. Both azide and cyanide can bring about complete inhibition of enzymatic cytochrome *c* oxidation.

3. Application of the mass law to the action of these substances at various pH values demonstrates that in both cases a complex is formed with the undissociated acids, HN_3 and HCN, while combination with ferricytochrome *c*, ferrihemoglobin, and ferrimyoglobin occurs with the respective ions. The dissociation constant for the enzyme-inhibitor complex was found to be 7×10^{-7} for hydrazoic acid and 5×10^{-7} for hydrocyanic acid.

4. The effect of azide on the oxygen uptake of stimulated frog muscle is dependent on the pH of the medium in qualitatively the same manner as the oxidation of ferrocytochrome *c* is in a cell-free homogenate.

5. The *in vitro* findings are compared with the action of azide and cyanide on intact tissues and microorganisms.

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MICROBIOLOGICAL ASSAY OF CORN STEEP LIQUOR FOR AMINO ACID CONTENT

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The corn wet milling industry has been in operation for about 100 years in this country. Until large scale production of penicillin was started during recent years, all of the steep water, a by-product of the wet milling process, was disposed of as feed. Since this by-product has played an important rôle in the production of penicillin, a systematic analytical evaluation of the nitrogenous components, such as that recently carried out by White *et al.* (1), is in order. In these studies, some amino acids were substituted for steep water with satisfactory results. A reasonably complete amino acid assay might indicate that certain other amino acids present in steep water are responsible for the well known stimulation of *Penicillium* by steep water. Accordingly, hydrolyzed and unhydrolyzed preparations were assayed for amino acids and a few other components.

Preparation of Samples

The steep water used for the assay of amino acids was "heavy" steep water which had been incubated and then concentrated by evaporation to 54.2 per cent of dry solids and was taken from a tank car lot destined for use in the production of penicillin. Hydrolyzed samples were prepared by refluxing 1 gm. of steep water (dry basis) in 40 ml. of 6 N HCl for 18 hours. Most of the acid was removed by distillation and the remainder was neutralized with 5.0 N NaOH. The hydrolysate was then filtered and adjusted to the desired volume. The unhydrolyzed samples were diluted with water after being adjusted to pH 6.8 with 6.0 N sodium hydroxide and were not filtered, since representative samples of the nitrogenous material for amino acid assay were wanted. Aliquots of both the hydrolysate and the steep water suspension were diluted to the desired volumes for assay.

The general analytical data for the concentrated steep water are presented in Table I. The total nitrogen was determined by the Kjeldahl method (2). Amino nitrogen was determined by the Van Slyke method (3), and a procedure described by Friedemann and Graeser (4) was followed for lactic acid determination.

1 kilo of this sample of steep water, dry basis, is the concentrated extract from 13 to 16 kilos of corn.

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Assay Procedures

The amino acids used to prepare standard solutions for the work reported were as follows: DL-leucine (Merck), DL-isoleucine (Merck), L-glutamic acid (Pfanstiehl), DL-valine (Lemke), DL-threonine (Merck), L-lysine dihydrochloride (Lemke), L-arginine hydrochloride (Lemke), L-histidine (Lemke), L-proline (Merck), DL-phenylalanine (Lemke), DL-methionine (Merck), DL-aspartic acid (Monsanto), L-cystine (Pfanstiehl), DL-serine (Lemke), L-tyrosine (Merck), and DL-alanine (Merck). Solutions of these amino acids were kept under toluene in the refrigerator.

The cultures were carried on yeast-dextrose-veal infusion agar. Subcultures were made at weekly intervals and incubated for 24 hours at 37° and then stored in the refrigerator. The standard microbiological techniques of inoculation (5–7) and incubation at 37° for 72 hours were followed.

The acid produced was titrated in the culture tubes with 0.05 N NaOH to pH 7 with bromothymol blue as an indicator. The liquid was stirred with air bubbles from a capillary tube. Each amino acid assay was carried out twice in triplicate for each assay level. Each value reported in this paper is derived from five levels. The results are shown in Table II.

TABLE I

Per Cent Composition of "Heavy Incubated" Steep Water

Dry substance	54.2
Total N, dry basis	8.7
Ash, dry basis	20.6
Amino N, before hydrolysis	2.9
" " after "	7.0
pH	3.7
Lactic acid, dry basis	26.0

Leucine, isoleucine, valine, and glutamic acid were determined with *Lactobacillus arabinosus* 17-5. The medium used is described by Shankman (5).

Threonine was determined with *Streptococcus faecalis*. The basal medium of Shankman (5) was used with the exception of the folic acid concentrate, which was increased 10-fold.

Lysine, arginine, proline, phenylalanine, histidine, methionine, cystine, serine, alanine, and aspartic acid were determined with *Leuconostoc mesenteroides* P-60. The medium used differed from Dunn's Medium D (7) in the following respects: Ammonium sulfate was used instead of ammonium chloride and the amount of histidine was increased 5-fold. No hydroxyproline was used. For the first five amino acids in this group, the folic acid (Wilson Laboratories) was increased 1000 times.

The amino acid assays of steep water in Table II are of interest for several reasons. First, the total nitrogen content is almost completely accounted for by ammonia and amino acids. Second, the large proportion of the nitrogen represented by alanine, glutamic acid, and arginine is of interest. The alanine content is surprising, since it accounts for more than one-fourth of the total nitrogen.

The amino acid values are presented for consideration by those who use steep water in microbiological substrates or as a source of certain amino acids. For example, the use of steep water made possible the commercial production of penicillin. This may be due to the presence of certain amino acids in steep water. The increase in the amino nitrogen upon hydrolysis from 2.9 to 7 per cent suggests that not all the amino acids found in the hydrolysate are present as such before hydrolysis. Further evidence for this belief is the lower amount of nitrogen accounted for by the four amino acids assayed in the unhydrolyzed steep water (Table II). The presence of peptides is indicated.

TABLE II

Amino Acid Assays of Corn Steep Water

Amino acid	Per cent nitrogen accounted for	
	Hydrolysate	Unhydrolyzed steep water
Leucine	5.86	4.80
Isoleucine	3.40	2.80
Valine	3.88	2.86
Glutamic acid	7.97	3.00
Threonine	3.44	
Lysine	3.97	
Arginine	8.23	
Histidine	6.78	
Proline	4.75	
Phenylalanine	2.05	
Methionine	1.07	
Aspartic acid	1.68	
Cystine	1.22	
Alanine	27.70	
Tyrosine	0.74	
Ammonia*	12.70	
Total	95.44	

* Determined by distilling a portion of neutralized hydrolysate for 3 minutes in the presence of a 10 per cent suspension of $Mg(OH)_2$.

SUMMARY

Amino acids determined microbiologically together with ammonia account for over 95 per cent of the total nitrogen in concentrated steep water.

After hydrolysis, more than one-quarter of the nitrogen is present as alanine. Although arginine accounted for the next highest percentage of nitrogen, the glutamic acid content in terms of gm. of amino acid per gm. of steep water is higher. Unhydrolyzed steep water yields significantly lower apparent amino acid values than does the hydrolysate. This suggests that some of the potential amino acids are present as amino acid residues in more complex structures, possibly polypeptides.

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URINARY EXCRETION OF AMINO ACIDS BY HUMAN SUBJECTS ON NORMAL DIETS*

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(Received for publication, October 11, 1947)

In previous studies (1–4) we have reported on the microbiological assay of apparent free amino acids in dog and human plasma, sweat, and cerebrospinal fluid. As a further extension of our studies on body fluids, we are presenting a study of the urinary excretion of fifteen amino acids in the free and combined form on eighteen normal human subjects on their usual dietary régime.

EXPERIMENTAL

Methods—The subjects were five female and thirteen male freshmen medical students, who collected 24 hour urine specimens under toluene. The body weights of these subjects ranged from 110 to 183 pounds, with a mean of 147 pounds. The subjects were on their usual dietary régime which gave urinary excretion values for total nitrogen, ranging from 6.3 to 18.4 gm. per day with a mean of 12.5 gm. The caloric intake ranged from 1372 to 3582 calories per day with a mean of 2129 calories. Urine volumes ranged from 1241 to 4100 ml. per day with a mean of 1840 ml.

The microbiological methods for the assay of arginine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine, and valine were those described previously by Hier and Bergeim (1, 2) and by Hier *et al.* (5), except that threonine and methionine were assayed with *Streptococcus faecalis* according to Stokes *et al.* (6). Tryptophan values were obtained by the method of Dunn *et al.* (7). Cystine and aspartic acid were assayed with *Leuconostoc mesenteroides* P-60 with the improved Medium D described by Dunn *et al.* (8, 9). Proline was assayed with *Leuconostoc mesenteroides* P-60 with the medium of Barton-Wright *et al.* (10).

Small tubes (13 × 100 mm.) were used and only 1 ml. of basal medium was added to each tube. After the addition of standard and unknown solutions, the final volume was adjusted to 2 ml. and the tubes were covered

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with aluminum caps and sterilized for 10 minutes. All other technical details have been previously described (1), except that the tubes were titrated with 0.02 N NaOH.

For the assay of the apparent free amino acids, the diluted urine was used. Total amino acids, except tryptophan, were determined on acid-hydrolyzed urines. Total tryptophan was determined on alkali-hydrolyzed urines.

25 ml. of urine were made 2 N with 1:1 H_2SO_4 (approximately 3.2 ml.) and diluted to 30 ml. 5 ml. of urine were made 2 N with 12 N NaOH (approximately 1 ml.). The samples were heated in the autoclave at 15 pounds pressure for 5 hours. The tubes were cooled and the hydrolyzed urine adjusted to pH 6.8, filtered, and diluted to the desired volume.

Effect of Urinary Urea—In earlier studies on the assay of apparent free amino acids in urine, we found a definite inhibition of growth of assay organisms at the dilutions used. Since it has been shown by various workers (11–13) that urea is bacteriostatic and bactericidal for a number of Gram-negative and Gram-positive bacteria, we investigated the effect of various concentrations of urea on *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* to determine at what concentration urea inhibition began.

Inhibition of growth began at approximately 3 mg. of urea per assay tube. This growth inhibition was greater when the urea-containing solution was autoclaved at 15 pounds pressure for 10 minutes than when the urea solution was sterilized by filtration through a Berkefeld filter. There is some increase in pH upon autoclaving, but this was not great enough to account for the growth inhibition. It appears from these experiments that urea or ammonia, or both, is responsible for most of the growth inhibition in microbiological assays of urine. Accordingly, to minimize inhibition, dilutions or aliquots of urine were used in most cases containing less than 3 mg. of urea per tube. In the hydrolyzed urine specimens, there was no inhibition, since the urea was decomposed in the autoclaving process. Dunn *et al.* (14) also found that the acid hydrolysis of urine was an effective treatment for the determination of total amino acids.

DISCUSSION

Values are reported for the fifteen amino acids in mg. per 24 hours in Table I as apparent free amino acids, total amino acids, combined amino acids, and the percentage of the total in the combined form.

It is seen that the amino acids excreted by the different subjects show no significant variations in amount, although there is a wide variation in the type of "normal" diets, caloric intake, urine volume, and total nitrogen excretion. Analyses were also made for uric acid, creatinine, and ammonia. The results are not included in the tables, since no significant correlation could be observed.

Apparent free amino acids were present in every case except for aspartic acid. Only one specimen contained apparent free aspartic acid. Two subjects did not excrete any apparent free glutamic acid.

TABLE I

Urinary Excretion of "Free" and "Combined" Amino Acids by Eighteen Subjects on Normal Diets

Reported as mg. per 24 hours.

	Free		Total		Combined		Per cent combined form of total	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Arginine	11.0–36.2	21.3 ±6.9	12.2–45.0	23.7 ±7.9	0–13.8	6.6	0–52.9	24.2
Aspartic acid	0–23.6	1.3 ±0.6	87.4–258.8	164.5 ±46.7	82.7–258.8	163.2	82.1–100	99.2
Cystine	45.2–138.0	87.7 ±25						
Glutamic acid	0–63.7	35.8 ±19.2	102.4–769.5	351.4 ±151.4	72.6–708.7	315.6	79.0–100	89.8
Histidine	60.3–378.0	188.3 ±99.2	65.4–438.8	203.3 ±101.1	5.1–123.2	40.5	4.8–34.5	14.9
Isoleucine	0–20.1	5.9 ±4.5	11.8–33.4	20.3 ±5.5	3.9–30.3	14.4	18.3–100	71.0
Leucine	3.8–18.5	9.6 ±3.3	11.9–40.0	21.2 ±6.6	3.5–29.6	12.3	29.4–81.8	55.6
Lysine	18.2–88.2	33.6 ±16.9	35.6–166.0	73.2 ±29.4	15.4–77.8	43.0	23.4–74.8	57.1
Methionine	4.1–13.5	7.8 ±2.9	4.1–15.0	8.6 ±2.8	0.5–6.3	2.3	4.7–60.5	24.6
Phenylalanine	7.5–34.0	16.4 ±7.1	10.3–45.4	23.3 ±7.9	3.1–16.9	8.4	14.4–66.6	35.2
Proline	3.7–14.8	8.5 ±2.9	23.1–62.1	42.8 ±12.9	18.1–54.4	34.3	65.1–89.0	80.2
Threonine	12.4–49.5	24.4 ±10.9	14.8–84.4	53.8 ±19.5	18.8–53.8	31.3	41.7–67.9	58.0
Tryptophan	8.5–56.0	24.6 ±11.3	11.2–86.1	41.4 ±17.5	0.6–28.3	18.4	1.3–71.8	41.9
Tyrosine	10.5–43.9	20.8 ±1.7	23.4–100.3	52.5 ±18	5.9–60.3	31.7	25.2–75.1	60.4
Valine	0–7.5	4.5 ±2.2	11.0–30.0	19.8 ±5.6	6.1–28.2	15.3	55.4–100	77.3

Values are reported for apparent free cystine only, as the values for total cystine were consistently lower after acid hydrolysis, apparently because of partial destruction.

With regard to the other amino acids determined, it is believed that most of the amino acids are stable under the conditions of our experiments.

There is nevertheless a possibility that some racemization may have occurred in certain cases during hydrolysis in the autoclave. Only the optically active forms of the amino acids under consideration are determined by the microbiological methods.

Certain of the amino acids are excreted mainly in the combined form. Thus aspartic acid is present to the extent of 99 per cent in the combined form, glutamic acid 90 per cent, isoleucine 71 per cent, proline 80 per cent, and valine 77 per cent. Steele *et al.* (15) also have reported that aspartic acid exists in human urine almost entirely in a bound form. High values were found for total glutamic acid, aspartic acid, and histidine. Intermediate values for total lysine, threonine, tyrosine, proline, and tryptophan were found. Values for total arginine, isoleucine, leucine, phenylalanine, valine, and methionine were low.

TABLE II

Comparison of Average Microbiological Values of Urinary Amino Acid Excretion Reported as mg. per 24 hours.

	Normal diet		Free form from Steele *et al.* (15)			Total, from Dunn *et al.* (14)	
	Free form	Total	Egg	Boiled soy bean	Auto-claved soy bean	Normal diet	K ration
Arginine	21.3	23.7	4.4	5.1	8.1	35.6	41.5
Aspartic acid	1.3	164.5	0	0	0	190.0	219.9
Cystine*	87.7		76.2	68.7	74.0		
Glutamic acid	35.8	351.4	61.0	33.2	49.0	341.3	359.1
Glycine						463.0	1277.7
Histidine†	188.3	203.3	74.6	73.2	69.3	188.5	251.6
Isoleucine	5.9	20.3	5.0	4.5	4.5	19.3	19.5
Leucine	9.6	21.2	0	0	13.0	31.2	39.1
Lysine	33.6	73.2	1.5	3.0	1.5	83.1	75.7
Methionine	7.8	8.6	2.4	2.5	4.3	11.9	22.0
Phenylalanine	16.4	23.3	4.9	7.1	3.2	32.6	31.8
Proline	8.5	42.8	7.0	7.0	15.0		
Serine			27.0	21.3	26.5		
Threonine	24.4	53.8	24.9	19.7	22.0	57.8	65.9
Tryptophan‡	24.6	41.4	7.5	7.5	8.4		
Tyrosine	20.8	52.5	16.5	11.0	14.2		
Valine	4.5	19.8	7.3	4.1	9.2	2.9	36.6

* 100 mg. on normal diet, C and K ration (16).

† 250 mg. on C ration, 200 mg. on K ration, and 175 mg. on normal diet (16).

‡ 12 to 30 mg. on normal diet (17), 6.9 mg. on egg or soy bean (18), 20 mg. on C ration and normal diet, 40 mg. on K ration (16).

Although it is possible that many of the combined urinary amino acids are present in peptide combination, it also appears possible that part of the amino acids, especially of those found largely in combined form, may exist in the urine as conjugates.

A comparison of our average microbiological values with those of other investigators is given in Table II. Our values for free amino acid excretion are higher in most cases than those reported by Steele *et al.* (15) for subjects on a diet containing either egg or soy bean as the main source of protein. We also found leucine present both in the free and combined form. Our values for total amino acids agree quite closely with those of Dunn *et al.* (14) except in the case of valine. They found lower values than ours on their normal diet. However, the total valine excreted by their subjects on Army K ration was higher than on their normal diet.

SUMMARY

The amounts of apparent free and combined forms of fifteen amino acids have been determined by microbiological methods in 24 hour urines of five normal females and thirteen normal male subjects on their usual dietary régime.

All the apparent free amino acids were found in most of the urine samples except for aspartic acid. Two subjects did not excrete any apparent free glutamic acid.

Certain of the amino acids are excreted mainly in the combined form, such as aspartic acid of which 99 per cent is combined, glutamic acid 90 per cent, proline 80 per cent, valine 77 per cent, and isoleucine 71 per cent.

No significant correlation could be established between amino acid excretions and differences in urine volume, total nitrogen, uric acid, creatinine, or ammonia contents of the urines examined.

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CYTOCHEMICAL STUDIES OF MAMMALIAN TISSUES*

I. ISOLATION OF INTACT MITOCHONDRIA FROM RAT LIVER; SOME BIOCHEMICAL PROPERTIES OF MITOCHONDRIA AND SUBMICROSCOPIC PARTICULATE MATERIAL

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PLATES 2 AND 3

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During recent years, differential centrifugation has been employed fairly extensively for the isolation of cellular components that have been released from tissue cells by mechanical disruption of the cell membranes. One advantage of this procedure is that cell fractions may be obtained in sufficiently large amounts to allow extensive biochemical study. On the other hand, the possibility that artifacts may be produced when the cell membranes are broken must be seriously considered. These artifacts may take a variety of forms, including (1) morphological alteration of the intracellular components to such an extent that they are not readily identifiable with known cellular inclusions and (2) redistribution of biochemical properties as a result either of adsorption phenomena or of the loss of soluble substances from particulate material. Thus, in attempts to characterize biochemically a cellular component in the isolated state, it is of the utmost importance to demonstrate cytologically the integrity of the cellular component as well as the degree of homogeneity of the preparation.

By means of the technique of differential centrifugation a "large granule" fraction was isolated from rat liver (1). Concomitant biochemical studies showed that the respiratory enzyme systems, cytochrome oxidase and succinoxidase, were concentrated to a pronounced extent in this fraction (2, 3), a finding that made it imperative to identify cytologically the cellular component or components under study. In the latter respect, it was suggested that the large granule fraction consisted mainly of mitochondria inasmuch as the size of the granules corresponded closely to the size of mitochondria within the cell (1, 3). It was realized, however, that this evidence was not

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conclusive proof for the hypothesis that the granules were mitochondria, since the isolated granules did not possess two of the most important properties shown by the mitochondria of the hepatic cell, namely a predominantly rod-like shape and the ability to stain vitally with Janus green B.

As described in a preliminary report, the isolation of liver mitochondria that satisfied cytological criteria of integrity was accomplished by the use of hypertonic sucrose solutions (4) instead of the media (water and isotonic saline) that had been used in previous work (1–3). The present report includes a detailed description of the method of isolation of intact liver mitochondria and of some of the cytological, enzymatic, and chemical properties of these cellular elements. A description of certain properties of submicroscopic particles isolated from the cytoplasm of the liver cell is also included.

EXPERIMENTAL

Cytological Studies of Rat Liver Homogenates Prepared in Different Media

Effect of Water and Isotonic Saline—In previous experiments (1–3), the centrifugal fractionation of liver involved the use either of water or of 0.85 per cent NaCl solution. The large granules freed by disruption of the liver cell in these two media were practically all spherical in shape. In isotonic saline, the granules appeared as refractive bodies 0.5 to 2 μ in diameter (Fig. 1), whereas in water they were much larger and paler (Fig. 2). In neither medium did they stain vitally with Janus green B. It was therefore immediately apparent that the large granule fraction isolated from saline or water homogenates of liver did not contain morphologically intact mitochondria and that the relative proportion of mitochondrial material present in the fraction could not be determined by cytological techniques.

An additional disadvantage in the use of saline lay in the fact that many of the large granules were seen to be agglutinated in small clumps, while many others were firmly attached to free nuclei. If the saline homogenates were allowed to stand at 0°, the extent of agglutination became progressively more pronounced. A number of experiments indicated that agglutination of large granules also occurred in the presence of other electrolytes, including KCl, K_2SO_4, and phosphate buffers, and interfered to a considerable degree in the centrifugal fractionation of liver homogenates.[1] Agglutination was not observed, however, in water homogenates.

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[1] When all the free nuclei were removed from saline homogenates of rat liver by means of low speed centrifugation, an irregular and usually great proportion (40 to 80 per cent) of the large granules, as shown by succinoxidase determinations, was found in the nuclear sediment. Attempts to improve the yield of large granules by resuspension and resedimentation of the nuclei were not successful, probably because of the tendency of the granules to agglutinate in the presence of salt. These experiments confirm and offer an explanation for observations previously reported (2).

Effect of Solutions of Sucrose—Although on cytological grounds neither otonic saline nor water was a satisfactory medium for the isolation of ntact mitochondria, both the above observations and experiments previusly reported (1, 3) indicated that large granules were better preserved orphologically in isotonic solutions than in water, the latter medium havng caused visible swelling of the granules. The agglutination caused by aCl and other electrolytes at the isotonic level, however, resulted in a rohibitive loss when attempts were made to isolate large granules free from uclei. A search was therefore instituted for an isotonic medium that ould not cause agglutination.

It was found initially that large granules in liver homogenates prepared in approximately isotonic solutions (0.25 M) of the non-electrolyte, sucrose, were not agglutinated and were roughly the same size as the corresponding elements seen in isotonic saline homogenates (Fig. 3). Preliminary centrifugation experiments with 0.25 M sucrose as the medium showed that it was possible to effect separation of nuclei and large granules without difficulty, the latter having been obtained in excellent yield.

In subsequent microscopic studies of rat liver homogenates prepared in sucrose solutions, it was noted that many of the free "large granules" were distinctly elongated in shape when the liver cells were disrupted in a hypertonic sucrose solution (0.5 M). A series of homogenates was then prepared in increasing concentrations of sucrose. It was evident that the number of rod-like forms was roughly proportional to the concentration of sucrose until an optimum concentration of 0.8 to 1.0 M was attained. A sucrose concentration of 0.88 M was chosen for further cytological studies and for experiments in centrifugal fractionation.

Microscopic examination of homogenates of rat liver prepared in 0.88 M sucrose (Fig. 4) revealed an enormous number of refractive bodies of relatively uniform diameter (approximately 0.3 to 0.5 μ). There was considerable variation in the length of the bodies, 50 to 75 per cent of them being 1 to 5 μ in length and the remainder being either slightly elongated or granular. They were readily stained when a solution of Janus green B in 0.88 M sucrose (dye concentration 1:10,000 to 1:20,000) was introduced under the cover-slip. The staining reaction was fainter but perceptible when a dye concentration of 1:40,000 was used. They were not stained vitally by neutral red. On the basis of the morphological and staining characteristics, it was concluded that the bodies were intact mitochondria.

Occasional unbroken liver cells in 0.88 M sucrose homogenates were normal in appearance and remained so for several hours. The intracellular mitochondria were predominantly rod-like and filamentous and closely resembled the corresponding elements in the free state (Figs. 6, 7). The normal aspect of unbroken liver cells in this hypertonic medium was in sharp contrast, however, to the appearance of liver cells suspended in isotonic media. In homogenates prepared in 0.85 per cent NaCl solution, for example, the intracellular mitochondria very quickly became swollen and spherical (Fig. 8) and within a comparatively short time underwent disintegration.

Free nuclei in 0.88 M sucrose homogenates were practically structureless in appearance and closely resembled the nuclei of unbroken liver cells.

When the 0.88 M sucrose homogenates of rat liver were fixed by the addition of an equal volume of a 4 per cent aqueous solution of osmium tetroxide and the mixture allowed to stand for 24 hours at 4°, it was found possible to make smears that were suitable for staining by standard cytological techniques. It should be pointed out, however, that the procedure of fixation and smearing caused some of the elongated mitochondria to become distorted and others apparently to break. The smears (Fig. 4) thus showed occasional deformed mitochondria and, in general, contained a smaller proportion of long rods than was present in the unfixed homogenates. The fixed mitochondria were deeply stained by aniline acid fuchsin (Fig. 4) according to the Altmann method (5), by hematoxylin, by such basic dyes as safranine and crystal violet, and were Gram-positive when stained by the Gram technique.

Preliminary centrifugation experiments showed that the mitochondria could be sedimented and washed with 0.88 M sucrose without appreciable morphological change (Fig. 5). The washed mitochondria retained the ability to stain vitally with Janus green B at dye concentrations as low as 1:20,000 to 1:40,000.

Procedure for Centrifugal Fractionation of Rat Liver Homogenates Prepared in 0.88 M Sucrose

Adult albino rats were used in the present study. The animals were given only water for 18 hours before each experiment. They were sacrificed by decapitation. The livers were removed and forced through a masher fitted with a 1 mm. mesh screen that retained the connective tissue framework and allowed the parenchymatous part of the organs to be collected as a pulp. The liver pulp was immediately placed on ice, and in all subsequent steps of the fractionation the temperature was maintained at 0–5°. In each experiment, 5 gm. of liver pulp were homogenized in an all-glass apparatus (6) in 0.88 M sucrose (Merck, reagent grade), the total volume of the homogenate being 50 ml.

Sedimentation of Nuclei, Intact Liver Cells, and Red Blood Cells—Separation of the nuclei, residual unbroken liver cells, and red blood cells was effected by centrifuging 40 ml. of the homogenate three times for 10 minutes at 600 × gravity. Calibrated tubes of 15 ml. capacity were used in the horizontal yoke of the International centrifuge. In the first of the centrifugations, each 10 ml. portion of the homogenate was carefully layered over 1 ml. of sucrose, thus forming a two-phase system. This procedure, which was possible because the density of the homogenate was slightly less than that of the sucrose solution, resulted in the sedimentation of nuclei and whole cells with very little loss in volume from the homogenate. The final yield of mitochondria was improved because these small elements did not tend to migrate through the interface at the low centrifugal force employed. The combined sediments, the first of which was by far the largest, were suspended in 0.88 M sucrose (Fraction N). This fraction contained red blood cells, residual intact liver cells, and free nuclei, most of the latter being aggregated in clumps of various sizes. Relatively few free mitochondria were seen. Only free mitochondria and occasional lipide droplets were visible when the supernatant or "liver extract" (Fraction E) was examined in the light microscope.

Isolation of Mitochondria—The liver extract, which contained the elements derived from the cytoplasm of the hepatic cells, was then centrifuged for 20 minutes at 24,000 × gravity. The supernatant (Fraction S_1) was retained for further fractionation. The firmly packed, opaque sediment of mitochondria was suspended in 35 to 40 ml. of 0.88 M sucrose. In order to effect purification and to free the preparation from the soluble substances of the original homogenate, the mitochondria were resedimented at 24,000 × gravity and resuspended in 0.88 M sucrose. The supernatant obtained after resedimentation of the mitochondria (Fraction Wm) was retained for analysis. The final suspension of mitochondria (Fraction Mw) was distinctly yellow in color and showed pronounced birefringence of flow when stirred.

The centrifuge[2] employed in the isolation of mitochondria was a motor-driven, self-balancing, angle type (inclination of tubes, 30° from the vertical) that was refrigerated and operated *in vacuo*. Lusteroid tubes of 50 ml. capacity were used in the isolation and resedimentation of mitochondria. The centrifugal force was calculated for the bottom of the tube.

Isolation of Submicroscopic Particulate Material—The supernatant (Fraction S_1) obtained after sedimentation of the mitochondria was submitted to 2 hours centrifugation at 41,000 × gravity, yielding a transparent, distinctly reddish pellet. The final supernatant (Fraction S_2), which was only faintly opalescent, was saved for analysis. The sedimented material was suspended in 0.88 M sucrose, resedimented by 2 hours centrifugation at 41,000 × gravity, and resuspended in the sucrose solution. The final suspension of submicroscopic particles (Fraction Pw) was opalescent, showed birefringence of flow on stirring, and was optically empty in the light microscope. The supernatant (Fraction Wp) obtained after resedimentation of the submicroscopic particulate material was also saved for analysis.

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[2] Constructed by J. Blum of The Rockefeller Institute for Medical Research.

Isolation of the submicroscopic particles was made with the angle centrifuge described above. Small lusteroid tubes (15 ml. in capacity, inside diameter 15 mm.) were used. From a consideration of the centrifugal force employed in the isolation of submicroscopic particles, the time of centrifugation, the viscosity and density of the sucrose solution, and the inside diameter of the centrifuge tube (7), it was estimated that most of the particulate material (density assumed to be 1.3) of a diameter of 100 mμ or greater would be sedimented. Recovery of material smaller than 100 mμ in diameter was probably not effected.

Chemical Methods

As shown in Table I, the fractions obtained by differential centrifugation of rat liver homogenates prepared in 0.88 M sucrose were analyzed for total nitrogen, succinoxidase activity, and pentose- and desoxypentosenucleic acid (PNA and DNA) phosphorus. Total nitrogen was estimated colorimetrically (8) after acid digestion of the samples. The determination of succinoxidase activity was carried out manometrically according to the method of Schneider and Potter (9), and the estimation of PNA and DNA phosphorus was made according to Schneider (10). A Beckman quartz spectrophotometer (model DU) was used in the colorimetric determinations.

Results of Chemical Studies

The results of studies of the distribution of nitrogen, nucleic acids, and succinoxidase activity in homogenates of rat liver prepared in 0.88 M sucrose solutions are summarized in Table I. When the total nitrogen, succinoxidase activity, and nucleic acid content of the various fractions were compared with the amounts originally present in the homogenates ($H = E + N = N + Mw + Wm + Pw + Wp + S_2$), it was evident that in each series of determinations the over-all recovery was satisfactory.

As shown in Table I, essentially all the DNA was recovered in Fraction N, which contained nuclei, residual unbroken liver cells, and red blood cells. This finding demonstrated that the removal of nuclei from the homogenate was complete. In one experiment the homogenate was strained through five layers of gauze. This procedure resulted in the removal of more than half of the free nuclei, as shown by a low DNA value (Table I). Since microscopic examination of Fraction N revealed the presence of relatively few free mitochondria, it was concluded that the

TABLE I

Distribution of Nitrogen, Succinoxidase, and Nucleic Acids in Rat Liver Homogenates Prepared in 0.88 M Sucrose

Preparation	No. of determinations	Nitrogen	Succinoxidase		Pentosenucleic acid phosphorus (PNA P)		Desoxypentosenucleic acid phosphorus (DNA P)	
		Total	Total (microliters O_2 per hr. $\times 10^{-3}$)	Q_{O_2} (microliters O_2 per hr. per mg. N)	Total	γ PNA P/mg. N	Total	γ DNA P/mg. N
		mg.			γ		γ	
H (homogenate)	4	134.6 (121.6*–146.0)	103.2 (95–114)	770 (650–870)	3050 (2540–3290)	22.7 (19.4–24.2)	1040 (528*–1310)	7.7 (4.4*–9.3)
E (liver extract)	4	100.1 (94.0–108.5*)	81.2 (72.0–90.2*)	810 (708–900)	2370 (1820–2850)	23.5 (19.4–26.3)		
N (nuclei and residual intact liver cells)	3	30.3 (12.2*–40.2)	19.0 (11.5*–25.8)	630 (511–940*)	575 (226*–830)	18.9 (18.5*–20.5)	1031 (524*–1400)	34.1 (29.2–43.8*)
Mw (mitochondria, sedimented twice)	4	35.0 (32.0–39.6)	74.2 (65.8–86.8)	2120 (1920–2340)	544 (430–623)	15.5 (13.1–17.4)		
Wm (supernatant after resedimentation of mitochondria)	4	8.8 (8.4–(9.1)	2 (*Ca.*) (1–3)	200 (*Ca.*) (100–300)	362 (279–414)	41.1 (31.0–48.7)		
Pw (submicroscopic particles, sedimented twice)	2	11.9 (11.2–12.6)	<1	<100	695 (591–798)	58.0 (52.5–63.5)		
Wp (supernatant after resedimentation of submicroscopic particles)	2	5.1 (4.9–5.38)			175 (166–183)	34.0 (34.0–34.0)		
S_2 (final supernatant)	3	41.9 (38.4–45.0)	<1	<30	643 (565–695)	15.3 (14.7–15.7)		

* In this experiment, the homogenate was filtered through five layers of gauze before fractionation. The marked figures are those occurring as extremes as a result of this procedure.

succinoxidase activity of the fraction was largely due to unbroken liver cells.

Further consideration of the data in Table I made it apparent that two fractions, the mitochondria (Fraction Mw) and the submicroscopic particles (Fraction Pw), were of particular interest from a cytochemical standpoint. The former contained a large proportion of the succinoxidase activity of the original homogenate but a relatively small amount of the PNA. The latter showed no appreciable succinoxidase activity but in terms of nitrogen content contained a large amount of PNA. It was also evident, on examination of the PNA P:N ratio obtained for Fraction Wm, that the initial sedimentation of the mitochondria had included submicroscopic particles. Although a separation of the two components was obtained on resedimentation of the mitochondria, it was not possible from the data in Table I to determine the degree of separation, and thus to establish the PNA content of mitochondria.

TABLE II

Succinoxidase Activity and Pentosenucleic Acid Content of Rat Liver Mitochondria

Preparation	Nitrogen		Succinoxidase			Pentosenucleic acid phosphorus (PNA P)		
	Total	Recovery	Total (microliters O_2 per hr. $\times 10^{-3}$)	Q_{O_2} (microliters O_2 per hr. per mg. N)	Recovery	Total	Recovery	γ PNA P/mg. N
	mg.	*per cent*			*per cent*	γ	*per cent*	
M (mitochondria, sedimented once)	43.8	(100)	69.2	1580	(100)	960	(100)	21.9
Mw_1 (mitochondria, sedimented twice)	33.8	77	67.8	2010	98	475	50	14.1
Mw_2 (mitochondria, sedimented 3 times)	30.6	70	65.0	2120	94	378	39	12.4
Mw_3 (mitochondria, sedimented 4 times)	24.0	55	52.6	2190	76	309	32	12.9

Accordingly, mitochondria were isolated from rat liver and suspended in 0.88 M sucrose according to the fractionation procedure described above. The mitochondria were then submitted to three cycles of sedimentation with intervening resuspension in 0.88 M sucrose solution. The original suspension and a portion of the suspensions of mitochondria obtained after each sedimentation were analyzed for nitrogen, PNA, and succinoxidase content. The analytical data are shown in Table II. It can be seen that after the first sedimentation there was a rise in succinoxidase Q_{O_2} and a fall in the PNA P:N ratio, but after subsequent sedimentations the values remained constant within the limits of experimental error. The total succinoxidase activity recovered in the preparations declined somewhat on repeated sedimentation, particularly after the final centrifugation. A consideration of the total nitrogen and nucleic acid phosphorus values indicated, however, that the loss in succinoxidase was due to incomplete sedimentation of whole mitochondria. Thus when the Q_{O_2} and micrograms of PNA P to mg. of N were calculated by difference for the supernatant after the final sedimentation, values of 1900 and 10.4, respectively, were obtained. These values were sufficiently similar to the corresponding figures characteristic of mitochondria (2190 and approximately 13) to indicate that repeated sedimentation and resuspension did not result in appreciable release of soluble, nitrogen-containing compounds from the mitochondria.

DISCUSSION

Cytological and Biochemical Characteristics of Isolated Mitochondria

Integrity of Mitochondria in Isolated State—Cytological studies summarized previously (4) and described in some detail in the present report offer good evidence for the morphological integrity of mitochondria obtained from rat liver homogenates prepared in 0.88 M sucrose. In the isolated state (Fraction Mw, Table I), the mitochondria had the same, usually elongated, shape possessed by mitochondria both within and outside cells in the homogenate and were morphologically similar to those seen in sections of liver fixed and stained by special cytological techniques (11, 12). The rather pronounced pleomorphism of the isolated elements was not surprising in view of the fact that pleomorphism is a characteristic property of the mitochondria of most tissues, including liver. Furthermore, the isolated mitochondria were stained vitally by Janus green B at very low dye concentrations. For many years, it has been recognized that Janus green B is a specific vital stain for intracellular mitochondria (11, 13). After fixation by appropriate methods, the isolated mitochondria also showed the staining reactions characteristic of intracellular mitochondria observed in similarly fixed and stained sections.

Finally, it should be noted that the isolated mitochondria satisfied chemical as well as the above morphological criteria of integrity. Thus their content of nitrogen, succinoxidase, and PNA remained constant on repeated sedimentation and resuspension in 0.88 M sucrose (Table II).

Homogeneity of Suspensions of Isolated Mitochondria—Perhaps the most difficult problem arising in the present studies was the establishment of the degree of homogeneity of the preparations of resedimented mitochondria (Fraction Mw, Table I). According to Claude (1), large granule preparations isolated from saline extracts of rat liver consist of a mixture of mitochondria and secretory granules. Other investigators, including Lazarow (14) and Hoerr (15), have stated, however, that the large microscopic granules within the liver cell correspond to mitochondria and thus that preparations of large granules isolated from liver consist practically exclusively of mitochondria. In this respect, it was found that homogenates prepared in 0.88 M sucrose offered an excellent opportunity both for the demonstration of secretory granules within the intact liver cell and for a determination of their fate after cell rupture and during centrifugal fractionation. When a freshly prepared homogenate in 0.88 M sucrose of liver from a fasted animal was stained vitally with a solution of neutral red (1:5000) in 0.88 M sucrose, two types of intracellular inclusion were stained selectively by the dye. These included a number of small spherical granules (*circa* 0.5 to 1 μ in diameter), most of which were localized at the peripheral margin of the cells, and occasional larger droplets (2 to 3 μ in diameter), which lay in the interior of the cells. The former were considered, on the basis of current cytological criteria, to represent true secretory granules and probably to contain components of bile; the latter were apparently lipide droplets, composed predominantly of phospholipide, that accumulate in liver cells as a result of fasting. The rod-like intracellular mitochondria remained unstained and greatly outnumbered the stained granules.

On examination of the extracellular elements in these homogenates, it was noted that the proportion of granules stained with neutral red was considerably less than the proportion stained within unbroken cells and that most of the stained extracellular elements corresponded in size to the relatively large lipide droplets. This finding indicated that many of the secretory granules did not exist as formed elements after rupture of the cell membrane. Finally, when the preparations of isolated and resedimented mitochondria (Fraction Mw, Table I) were stained vitally with neutral red, none of the elements in the suspensions took up the dye. Further examination of the fractions obtained by differential centrifugation of the liver homogenates showed that the material capable of staining with neutral red migrated centripetally during the initial sedimentation of the mitochondria. It was therefore concluded that the suspensions of resedimented mitochondria contained no secretory granules of the type demonstrable by staining with neutral red.

The most important evidence attesting to the absence of a significant number of extraneous components of relatively large size in the preparations of isolated mitochondria (Fraction Mw, Table I), and thus to the homogeneity of this fraction, was the finding that all the visible elements in the suspensions were stained vitally by Janus green B and after fixation by aniline acid fuchsin (5).

Submicroscopic particles constituted another possible source of contamination that would obviously escape detection on examination of the mitochondrial fraction with the light microscope but could, however, be detected by other means. Thus the average micrograms of PNA P to mg. of N ratio obtained for mitochondria sedimented once (Fraction M, Table II) was considerably higher than the corresponding values for repeatedly resedimented mitochondria (Fractions Mw_1, Mw_2, and Mw_3, Table II), indicating that submicroscopic material was present in Fraction M. The data in Table II clearly indicated, however, that one resedimentation was sufficient to remove the contaminating submicroscopic material.

Preliminary studies of the preparations of resedimented mitochondria in the electron microscope[3] also showed that little if any extraneous particulate material of small size was present (4).

Biochemical Characteristics of Liver Mitochondria—It seems reasonably certain from a consideration both of the chemical data (Tables I and II) and of the cytological observations that the respiratory enzyme system, succinoxidase, is associated with mitochondria. A large proportion (65 to 82 per cent) of the succinoxidase activity of the original homogenates of whole liver was recovered in the resedimented mitochondria, a negligible amount having been present in all other fractions with the exception of that containing nuclei and unbroken cells. The persistently high recovery of the enzyme in the mitochondrial fraction after repeated sedimentation made it extremely doubtful that this finding was simply the result of adsorption, particularly in view of the fact that succinoxidase is a complex system consisting of several components (16).

No attempt was made in the experiments reported to obtain a complete separation of nuclei and mitochondria through further fractionation of the suspension of nuclei and whole cells (Fraction N, Table I). In additional experiments, however, it has been possible by means of repeated homogenization and differential centrifugation of this fraction to obtain practically complete separation of DNA and succinoxidase activity with excellent recoveries of both, all but 1 to 2 per cent of the succinoxidase activity having been separated from the DNA. It can therefore be concluded that mitochondria contain all of the succinoxidase activity present in the cytoplasm of the liver cell and probably all of the succinoxidase activity present in the entire cell. Although cytochrome oxidase determinations were not carried out, it should be mentioned that this system is responsible for the oxygen consumption of the succinoxidase system and that the distribution of the two systems in fractions isolated from rat liver has previously (2, 3) been quantitatively identical.

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[3] The authors are indebted to Dr. K. R. Porter for his aid in the study of the fractions with the electron microscope.

It was also possible from the data in Table I to estimate the proportion of the total nitrogen and PNA of whole liver accounted for by mitochondria. Thus the amount of mitochondrial nitrogen in Fractions N and Wm was calculated from the total succinoxidase activity of these fractions and the succinoxidase Q_{O_2} of resedimented mitochondria. The total PNA due to mitochondria was then estimated from the micrograms of PNA P to mg. of N ratio characteristic of mitochondria (approximately 13, as shown by the data in Table II). On the basis of these calculations, the mitochondria were found to account for 34 and 19 per cent, respectively, of the nitrogen and PNA present in the original homogenate.

The mitochondrion can therefore be considered as a complicated functional unit possessing two of the most important respiratory enzyme systems of the cell and accounting for approximately one-third of the nitrogen and one-fifth of the PNA of whole liver. The presence of PNA in "large granules" isolated from saline extracts of rat liver was detected qualitatively by Claude (1), and quantitative measurements of the distribution of PNA in water homogenates of liver were reported by Schneider (2). The latter results are in good agreement with those presented in Table I. The biochemical significance of the PNA of mitochondria, particularly its possible relation to the respiratory enzymes, is a problem of great interest. In this respect, PNA was found to be present in crude solutions of succinic dehydrogenase prepared from liver mitochondria according to a method previously reported (17). In attempts to purify the enzyme by $(NH_4)_2SO_4$ fractionation, only partial separation of the nucleic acid and enzyme was effected, a considerable proportion of the former being retained in the enzymatically active fraction.[4] The question whether succinic dehydrogenase and PNA are actually related, however, can only be answered when the enzyme is obtained in a highly purified state. It is nevertheless tempting to believe that the "insolubility" of succinic dehydrogenase and other respiratory enzymes such as cytochrome oxidase is due to an association between the enzymes and highly polymerized PNA. It should also be pointed out that the PNA of mitochondria may account for the reaction of these cellular elements with the Gram stain in view of the demonstration that PNA is responsible for the Gram reaction in bacteria (18).

It is probable that the liver mitochondrion possesses many other enzymatic functions, including the oxidation of D-amino acids (19) and the dephosphorylation of adenosine triphosphate (2). It is certainly evident that much further work remains to be done before a complete picture of the biochemical nature of the mitochondrion will be established.

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[4] Hogeboom, G. H., and Schneider, W. C., unpublished experiments.

Mechanism of Effect of Concentrated Sucrose Solutions

The mechanism by which a strongly hypertonic solution of sucrose is capable of preserving the morphological and cytological characteristics of mitochondria is not fully understood, although studies to date have indicated that the effect is largely one of tonicity. Thus in a series of homogenates the proportion of rod-like forms progressively increased with increasing sucrose concentration. Conversely, when homogenates prepared in 0.88 M sucrose were diluted with water to a final sucrose concentration of 0.25 M (approximately isotonic to red blood cells) and were observed under the microscope, the elongated mitochondria were seen to become spherical within a few minutes. It should be pointed out, however, that the change from a rod-like to a spherical shape was apparently accelerated in a thin film on the microscopic slide because the mitochondria became spherical at a slower rate as long as they remained suspended in diluted homogenates. When the homogenates were prepared directly in 0.25 M sucrose, on the other hand, all the mitochondria were spherical within a few minutes (Fig. 3). The reason for this difference in behavior is not understood.

It was also noted that the effect of hypotonic media on mitochondria was at least to some extent reversible. Thus when an equal volume of 1.76 M sucrose was added to a water homogenate of rat liver, the large pale spherical bodies became much smaller and more refractive. Shrinkage of the large granules on addition of an equal volume of 1.76 M sucrose to isotonic saline homogenates was also observed.

The effect of various media on the mitochondria within intact liver cells was strikingly similar to the effect on the extracellular mitochondria. Unbroken liver cells in homogenates prepared in 0.85 per cent NaCl or 0.25 M sucrose contained swollen, spherical mitochondria (Fig. 8). In 0.88 M sucrose, however, the unbroken cells, despite severe oxygen deprivation, retained a normal appearance for hours when kept at 4° (Figs. 6, 7). The intracellular mitochondria remained elongated, and the cells showed no visible shrinkage in the hypertonic medium. The absence of any Brownian movement within the unbroken cells suggested that the cytoplasm had a gel-like consistency.

Some additional evidence that the effect of 0.88 M sucrose was due, at least in part, to the osmotic pressure of the solution was obtained in tests of other hypertonic media. Homogenates prepared in 3.0 per cent NaCl solution, for example, showed distinctly elongated extracellular mitochondria. When these preparations were allowed to stand, however, the mitochondria soon agglutinated and became granular. Maltose, at equimolar concentrations, had exactly the same effect as sucrose. Homogenates prepared in 0.88 M dextrose and mannose were similar to those prepared in 0.88 M sucrose, although the monosaccharides were not as effective as the disaccharide in maintaining the morphological stability of mitochondria over long periods of time. The trisaccharide, melezitose, also showed roughly the same effect as sucrose at equimolar concentration. The mitochondria quickly became spherical, however, in homogenates prepared in 0.88 M solutions of sorbose and the pentose, xylose.

It is recognized that factors other than the osmotic pressure of 0.88 M sucrose may well play an important rôle in maintaining the integrity of mitochondria and that the necessity for a relatively high osmotic pressure in the medium may reflect more than simply a control of the distribution of water within and outside the mitochondrial membrane. The osmotic pressure within the living hepatic cell at the mitochondrial membrane, on the other hand, is not known and may actually be higher than the blood osmotic pressure. In view of the above findings, particularly of the observations of intact liver cells in homogenates prepared in sucrose solutions, the possibility of existence of the latter situation should not be dismissed.

Characteristics of Submicroscopic Particles

Although the data in Table I showed that practically all the mitochondria except those present in unbroken liver cells (Fraction N) had been obtained sharply in a single fraction (Mw), it was apparent that the submicroscopic particles were distributed more widely. The micrograms of PNA P to mg. of N ratios for the various fractions indicated that the latter component of the cell was present in Fractions Wm and Wp as well as in Fraction Pw. By utilizing micrograms of PNA P to mg. of N values of 13, 58, and 15.3 for mitochondria, submicroscopic particles, and final supernatant, respectively, it was possible to calculate in terms of nitrogen the total amount of submicroscopic particles present in the fractions. Furthermore, in view of the observation that few free mitochondria were present in Fraction N, it could be assumed that the relative proportion of mitochondria and submicroscopic particles in this fraction was the same as in the liver extract (Fraction E). It was thus estimated that the submicroscopic particles contained approximately 20 per cent of the nitrogen and 50 per cent of the PNA of whole liver. It should be pointed out, however, that these figures are presented only as rough approximations, since they are based on several assumptions that may be somewhat in error.

It is therefore evident that a considerable proportion of the PNA of the liver cell occurs in sedimentable material that can be sharply differentiated from mitochondria. This material was smaller in size than mitochondria, contained much more nucleic acid in terms of nitrogen content, and showed essentially no succinoxidase activity, these properties being qualitatively characteristic of the "microsomes" studied previously (1, 3). The micrograms of PNA P to mg. of N values obtained in the analysis of Fraction Pw (Table I) showed that approximately 10 per cent of the total nitrogen of the submicroscopic particles was PNA nitrogen. Of some interest is the fact that this high PNA content falls well within the range of corresponding figures obtained for tobacco mosaic (20) and bushy stunt (21) viruses, both of which are said to be composed entirely, or almost entirely, of ribose nucleoprotein (22).

Another interesting property shown by the opalescent suspensions of submicroscopic particles (Fraction Pw, Table I) was birefringence of flow. This phenomenon has not been noted previously in suspensions of microsomes isolated from saline or water homogenates of liver and indicates that the particles in 0.88 M sucrose are asymmetrical in shape. Preliminary studies with the electron microscope[3] of the morphological characteristics of the elements in this fraction have, in fact, revealed the presence of an elongated fibrous component that is similar in structure to the endoplasmic material[5] seen in electron micrographs of cultured cells. It is possible that this asymmetrical particle may represent the postulated micellar structure of protoplasm supposedly responsible for sol-gel changes (14).

As mentioned previously, the relatively high density and viscosity of 0.88 M sucrose probably prevented complete sedimentation of the submicroscopic particles at the centrifugal force employed. It was therefore not possible to estimate what proportion of the nitrogen and PNA in the final supernatant (Fraction S_2) and thus in the liver cell was due to particles of a diameter less than 100 mμ. It should also be pointed out that the composition of the preparations of submicroscopic particles (Fraction Pw), in respect to the number and relative proportions of different components present, is not as yet known. Further studies of the fraction, isolated by sedimentation at higher centrifugal forces, are planned.

SUMMARY

1. The mitochondria of rat liver cells, when released by homogenization into water or isotonic NaCl solution, are altered to such an extent that they are not morphologically identifiable. When released into strong solutions of sucrose (0.8 to 1.0 M), however, they show the morphological and staining properties characteristic of normal intracellular mitochondria. Cytological studies of rat liver homogenates prepared in solutions of sucrose and other compounds at several concentrations indicate that the proportion of morphologically intact mitochondria, both extracellular and intracellular, increases with increasing concentration of solute and is related to the osmotic pressure of the medium. Sedimentation of mitochondria from homog-

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[5] Porter, K. R., in press.

enates prepared in 0.88 M sucrose and washing with the same medium does not result in any change in their morphological or staining properties.

2. A procedure for the centrifugal fractionation of rat liver homogenates prepared in 0.88 M sucrose is described. By this procedure it is possible to obtain mitochondria in suspensions that are apparently free from other cellular components. In the experiments reported, 65 to 82 per cent of the succinoxidase activity of the original homogenate was recovered in the mitochondria, the remainder of the enzyme activity being present in a fraction containing nuclei and unbroken liver cells. On the basis of cytological observations and additional chemical data, it is concluded that mitochondria contain all of the succinoxidase present in the cytoplasm of the liver cell and probably all of the succinoxidase present in the entire cell. It is further estimated that 34 per cent of the total nitrogen and 19 per cent of the pentosenucleic acid (PNA) of whole liver can be accounted for in mitochondria. The succinoxidase Q_{O_2} (microliters of O_2 per hour per mg. of nitrogen) and the ratio, micrograms of PNA phosphorus to mg. of nitrogen, are approximately 2100 and 13, respectively, for mitochondria. These values remain constant on repeated sedimentation and resuspension of mitochondria in 0.88 M sucrose.

3. Submicroscopic particulate material of a diameter of approximately 100 mμ or greater was also isolated from 0.88 M sucrose homogenates of rat liver by means of prolonged centrifugation at high speed. Suspensions of this material are opalescent, show birefringence of flow, and contain no succinoxidase activity. The PNA content of the submicroscopic particles is characteristically high, the ratio, micrograms of PNA phosphorus to mg. of nitrogen, being approximately 58 for this fraction. It is estimated that the submicroscopic particles account for approximately 20 per cent of the total nitrogen and 50 per cent of the PNA of whole liver.

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EXPLANATION OF PLATES 2 AND 3

PLATE 2

FIG. 1. Homogenate of rat liver prepared in 0.85 per cent NaCl solution. Fixed with OsO_4; stained with aniline acid fuchsin (5). The deeply stained large granules (diameter, 0.5 to 2 μ) are generally spherical in shape and tend to be agglutinated in small clumps. × 1100.

FIG. 2. Homogenate of rat liver prepared in water. Fixed with OsO_4; stained with 1 per cent aqueous solution of crystal violet. The large granules are swollen (diameter, 1 to 5 μ) and rather faintly stained but are not agglutinated. The granules in water homogenates do not stain with aniline acid fuchsin. × 1100.

FIG. 3. Homogenate of rat liver prepared in 0.25 M sucrose. Fixed with OsO_4; stained with aniline acid fuchsin (5). The large granules are approximately the same size as those in Fig. 1 but are not agglutinated. × 1100.

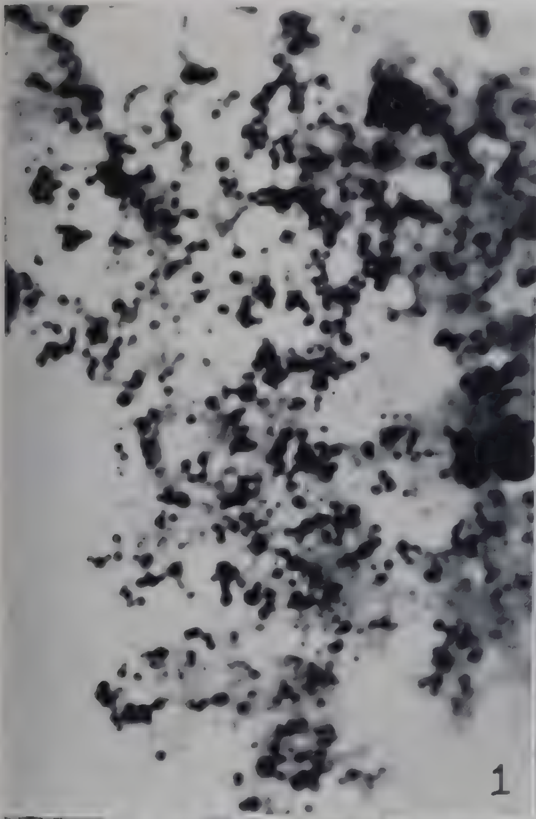
FIG. 4. Homogenate of rat liver prepared in 0.88 M sucrose. Fixed with OsO_4; stained with aniline acid fuchsin (5). Most of the large granules of Figs. 1 to 3 are distinctly elongated in shape and correspond morphologically to normal mitochondria. × 1100.

PLATE 3

FIG. 5. Mitochondria isolated by differential centrifugation from 0.88 M sucrose homogenate of rat liver (Fraction Mw, Table I). Fixed with OsO_4; stained with 1 per cent aqueous solution of safranine. The mitochondria have retained their elongated shape after isolation and resedimentation. × 1100.

FIGS. 6 AND 7. Unbroken liver cells in homogenate prepared in 0.88 M sucrose. The cells in Fig. 6 are unfixed and unstained; those in Fig. 7 have been fixed with OsO_4. The cells are normal in appearance; the intracellular mitochondria are predominantly rods and filaments (*cf.* Fig. 8). × 1100.

FIG. 8. Unbroken liver cells in homogenate prepared in 0.85 per cent NaCl solution. The cells are unfixed and unstained. The intracellular mitochondria are swollen and generally spherical in shape. This homogenate was prepared from the same liver as the homogenate shown in Figs. 6 and 7. × 1100.



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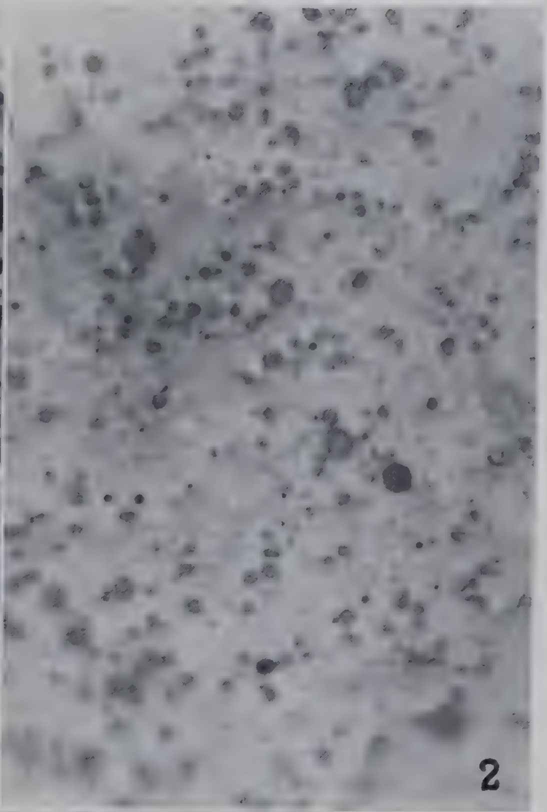

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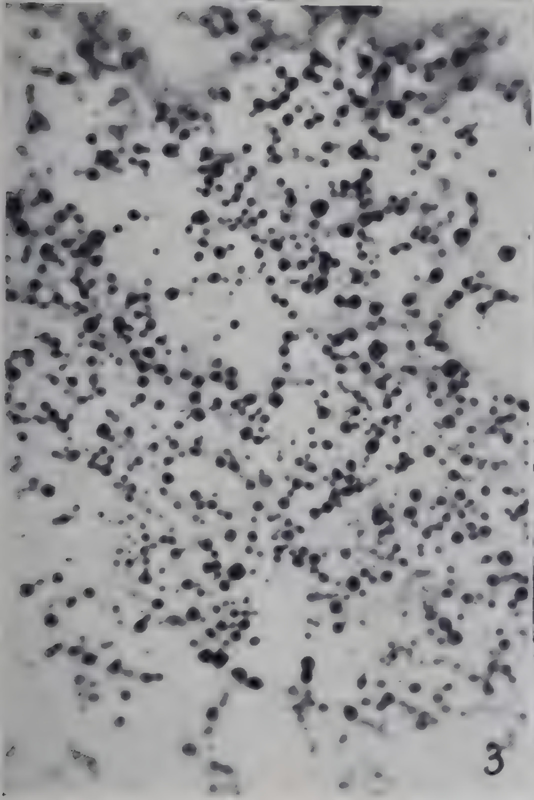

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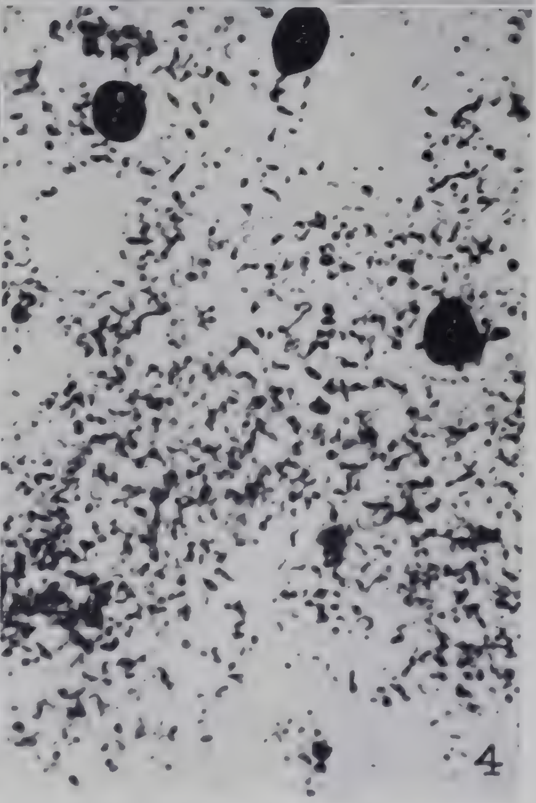

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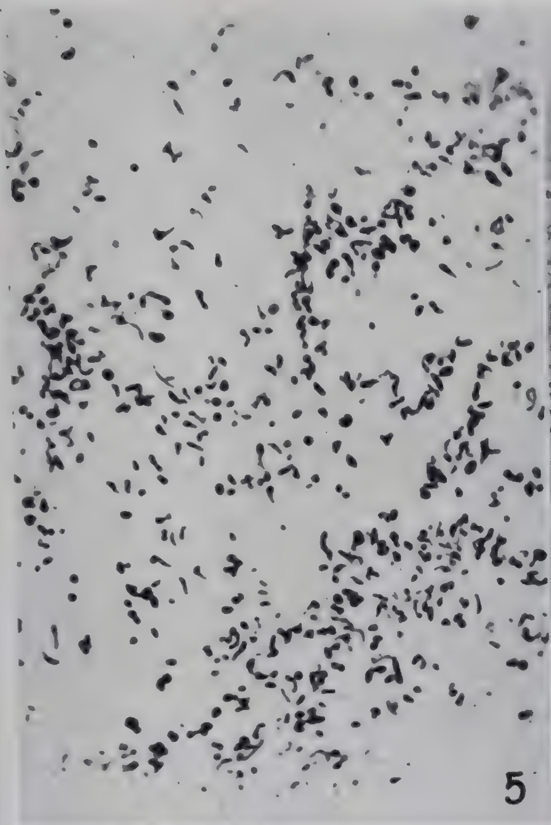

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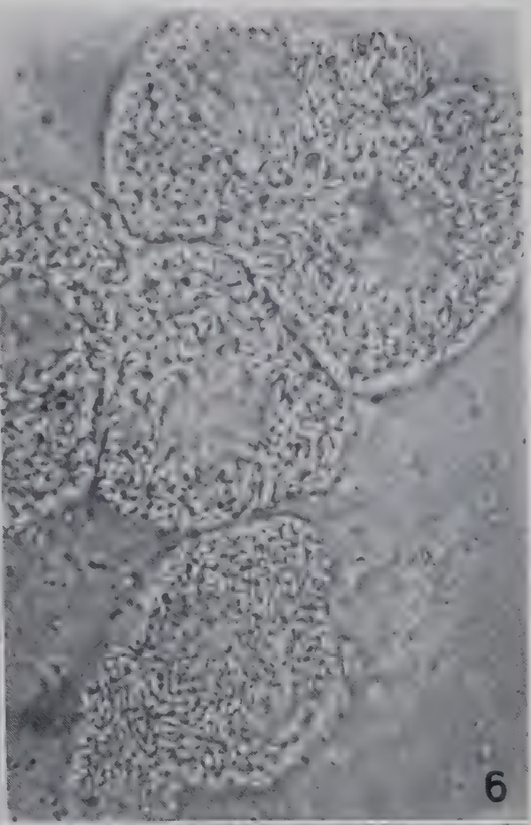

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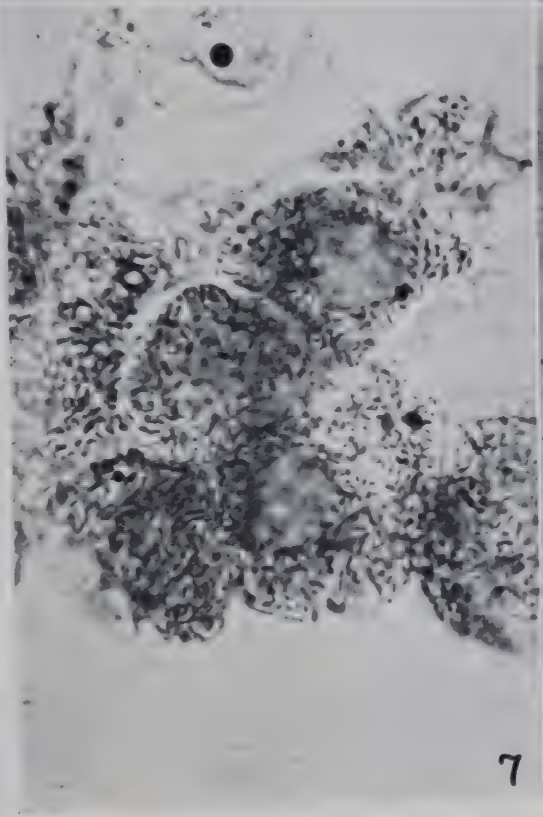

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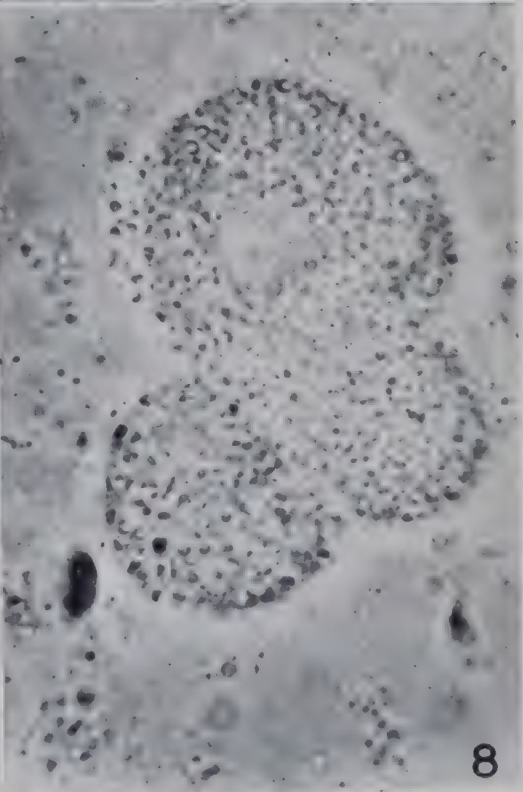

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(Hogeboom, Schneider, and Pallade: Cytochemical studies. I)

ARE PHOSPHOLIPIDES OBLIGATORY PARTICIPANTS IN FAT TRANSPORT ACROSS THE INTESTINAL WALL?*

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It is still a disputed question whether phospholipides are instrumental in carrying fats across the intestinal wall. By means of labeling agents it has been amply demonstrated that the small intestine is among the most active of tissues concerned with phospholipide metabolism. Studies with P^{32} in particular leave no doubt that not only during fat absorption is phosphate rapidly incorporated into phospholipide by the small intestine but phospholipide molecules likewise are continually renewed during the fasted state (1). The finding, therefore, that labeled fats during their absorption are incorporated into phospholipide molecules need not be interpreted as demonstrating that phospholipide formation is an essential step in fat transport. It is to be expected that whatever fatty acids are present in the intestinal wall will be drawn upon to furnish the building blocks for the phospholipide molecules. Indeed, the fact that during fat absorption the labeled fat is present in such large amounts would make them expected participants in phospholipide renewal (2–4).

In the present study, radioactive phosphorus has been employed to compare the rates at which phospholipides are turned over in the small intestine of the fasted and fat-fed animal.

On Use of Relative Specific Activity of Phospholipide Phosphorus As Measure of Phospholipide Turnover

It has been pointed out in a previous publication (5) that after the injection of radioactive phosphate into an animal a measure of the turnover rate of a compound in a tissue can be obtained from the "specific activity-time" relations of that compound and its immediate precursor. This procedure provides a method for determining the *absolute rate* at which the compound is synthesized and broken down, *i.e.* turned over. The use of this procedure is limited, since the immediate precursor is known for very few compounds and this is particularly true of the phospholipide molecule.

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* The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute of the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

If the precursor is not known, the use of specific activity (phospholipide P^{32})/(phospholipide P^{31}), in the measurement of turnover presents considerable difficulties because specific activity is influenced by many factors, some of which are difficult to control experimentally. For practical purposes these factors may be grouped according to whether they influence the numerator or denominator of the expression for specific activity.

Amount of Phospholipide P^{31}—The specific activity of the phospholipide fraction varies inversely with the amount of chemical phospholipide present in the tissue, since the latter is the denominator of the expression for specific activity.

Amount of Phospholipide P^{32}—The numerator of the expression for specific activity of phospholipide will depend upon (*a*) the rate of appearance and disappearance of phospholipide molecules and (*b*) the amount of P^{32} available for the formation of phospholipide at the site of synthesis.

The manner in which these factors influence the specific activity of phospholipide can be briefly stated as follows.

It has been repeatedly shown that the phospholipide content of the small intestine does not increase during fat absorption (6, 7). This has been confirmed in the present investigation (see Tables I to V). Hence, since we are interested in comparing the turnover rates of phospholipide of the small intestine in these two nutritional states, the amount of phospholipide P^{31} described above plays no part.

It is assumed that in the postabsorptive state the rate of synthesis equals the rate of breakdown of phospholipides in the small intestine. Since, as already noted above, no change in the phospholipide content of this tissue occurs after fat feeding, it may be concluded that the rate of appearance of the phospholipide in the fat-fed animal equals its rate of disappearance; presumably a steady state exists. It should not be inferred, however, that the turnover rates of phospholipides are the same in the fat-fed and in the fasted animals.

There is one more factor which influences the specific activity of phospholipide in the two states under consideration; namely, the specific activity of the P^{32} available for phospholipide synthesis. It is safe to assume that various phosphorus-containing compounds constitute a pool of P^{32} inside the cell available for phospholipide synthesis. The concentration of P^{32} in this pool will depend on such variable elements as permeability of the cell to P^{32}, rate of absorption of P^{32} from the site of injection, differences in body weight, and the relative avidity of the tissues for P^{32}.

In attempting to obtain a measure of the specific activity of the available pool, Hevesy and Hahn (8) first used the specific activity of intracellular inorganic phosphate (total minus extracellular). More recently Hevesy suggested the use of the labile phosphorus of adenosine triphosphate as quivalent to intracellular inorganic phosphorus, since the latter is in rapid quilibrium with the former.[1] Hevesy used the *ratio* of the specific activity f phospholipide phosphorus to specific activity of inorganic phosphorus as measure of turnover. This ratio he called *relative specific activity* (9).

Undoubtedly inorganic phosphate or labile phosphates provide an accuate index of the available pool, but because of the difficulty of measuring he intracellular phosphate the use of acid-soluble phosphorus for this urpose seemed entirely adequate. Hence, in this investigation the ratio f the specific activity of phospholipide phosphorus to the specific activity f total *acid-soluble phosphorus* in the same tissue was used as the relative pecific activity of phospholipide phosphorus.

It can be shown experimentally that, as an index of turnover, the use of elative specific activities instead of specific activities overcomes to a great xtent the variability from animal to animal (see variability in specific ctivities in Table III). The reason for this is obvious in view of the numerous factors which affect the availability of P^{32} for phospholipide synthesis. As has been pointed out, all these factors influence the specific activity of the phospholipides in the same way as they influence the specific activity of the phosphorus available for phospholipide synthesis. But the relative specific activity, being the ratio of the two, is not affected by these variables.

When the difference between the P^{32} in the available pool and the P^{32} present in phospholipide is greatest, a small increase in phospholipide turnover is reflected by a large change in the relative specific activity. This condition prevails quite early, long before the specific activity of phospholipide phosphorus reaches a maximum. The intervals at which turnover rates are compared below were selected in conformity with this consideration.

EXPERIMENTAL

Methods

Fat absorption as measured here represents the difference between the amount administered and the amount that remained in the small intestine at the end of the period of observation. The unabsorbed fat was washed out of the small intestine with acetone or ether and determined by the oxidative technique (10). The isolation and determination of radioactive phospholipide have been described elsewhere (11). Acid-soluble phosphorus of tissues was obtained as follows: The tissue was homogenized in 10 per cent trichloroacetic acid and centrifuged. An aliquot of the supernatant was mounted on a blotter for determination of P^{32}, whereas the total phosphorus was determined on a different aliquot by King's method (12).

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[1] Hevesy, G., personal communication.

Lipide Phosphorylation in Small Intestine of Dog

Phospholipide Formation in Different Parts of Small Intestine in Fasted Dog—Six dogs, fasted for 24 hours, were injected intravenously with radioactive phosphate. 6 hours later, the entire gastrointestinal tract was excised immediately after the animals had been anesthetized by an intracardiac injection of nembutal, and samples were taken as follows: The portion between the pylorus and the duodenal mesentery was taken as duodenum. The next 15 inches of intestine were discarded, but the following 12 inches were taken as representative of the jejunum. The 15 inches of intestine above the cecum were used as ileum. The phospholipide content of the *mucosa* of these three intestinal segments, the specific activities, and the relative specific activities of their phospholipide phosphorus are recorded in Table I (Dogs 1 to 6).

TABLE I

Phospholipide Formation by Mucosa of Duodenum, Jejunum, and Ileum in Fasted and Fat-Fed Dog

Tissues were excised 6 hours after P^{32} had been injected intravenously.

Dog		Phospholipide P^{31} per gm. dry weight			Specific activity of phospholipide P*			Specific activity of acid-soluble P*			Relative specific activity†			Treatment
No.	Weight	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	
	kg.	*mg.*	*mg.*	*mg.*	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$				
1	7.0	4.70	4.40	4.20	8.52	8.72	10.8							Fasted
2	7.5	4.85	4.31	4.56	14.3	10.7	11.6	42.0	33.8	29.3	0.34	0.32	0.40	
3	8.1		5.05	4.57	16.4	18.1	15.7	50.0	44.0	39.5	0.33	0.41	0.40	
4	5.7	5.67	5.90	5.68	13.2	11.8	14.5	18.9	15.1	26.7	0.70	0.78	0.54	
5	6.2	6.45	5.46	5.50	16.4	15.3	15.2	37.6	32.4	27.4	0.44	0.47	0.55	
6	10.2	5.20	5.05	5.05	8.02	10.1	8.75	24.6	26.4	21.4	0.33	0.38	0.41	
Average					12.8	12.5	12.8				0.43	0.47	0.46	
7	7.9	4.50	4.40	4.20	13.4	12.9	13.3							Cream-fed
8	6.5	4.60	4.90	4.20	12.3	13.8	13.0	42.0	39.0		0.29	0.35		
9	11.6	4.63	4.79	4.05	6.88	7.04	5.69	21.3	19.4	18.8	0.32	0.36	0.30	
10	7.1	6.05	6.10	5.07	16.2	14.6	11.6	32.7	27.4	24.4	0.50	0.53	0.48	
11	7.0	6.40	6.28	4.91	11.0	11.1	10.4	18.8	17.4	22.6	0.59	0.64	0.46	
Average					12.0	11.9	10.8				0.43	0.47	0.41	

* The specific activity is expressed as the per cent of the administered P^{32} per mg. of phosphorus.

† Relative specific activity is the ratio of specific activity of phospholipide P to that of acid-soluble P.

Table I shows that the phospholipide contents of the mucosa of duodenum, jejunum, and ileum are similar. Moreover, their relative specific activities are the same; the average values obtained were 0.43, 0.47, and 0.46. This means that the rates of turnover of phospholipide in these three parts of the small intestine are the same.

Phospholipide Formation in Different Parts of Small Intestine in Fat-Fed Dog—The second group of dogs (Dogs 7 to 11, Table I) was treated as above, except that they received 50 cc. of cream by stomach tube half an hour before the intravenous administration of radioactive phosphate. There can be no doubt that in the fat-fed dog, as in the case of the fasted animal, the phospholipide turnover in different parts of the mucosa of the small intestine is the same.

A comparison of the relative specific activities for the fasted and fat-fed dogs shown in Table I suggests that the passage of fat through the intestinal wall does not alter the rate of phospholipide turnover in any portion of the small intestine's *mucosa*. The variability of the values from dog to dog, however, precludes a definite conclusion as to whether phospholipides are obligatory steps in fat transport across the gut wall, and for this reason evidence was sought by another approach.

Separation by Ligature of Single Small Intestine into Fat-Absorbing Area and Control or Fasted Area—The dogs in Table II were subjected to a 24 hour fast. Under local anesthesia (procaine) a small abdominal mid-line incision was made and a ligature placed around the portion of small intestine immediately below the incision. In this way the lumen of the small intestine was divided. The entire procedure was carried out with a minimum of trauma and with no interference of blood supply to either portion of the gut. Fat was then usually injected into the duodenal side of the ligated intestine. Thus the duodenal side of the small intestine was made to absorb fat, whereas the ileal side remained in the fasted state. White lacteals were usually found on the duodenal side right up to the ligature, whereas the ileal side was devoid of any white ones. In some dogs the unabsorbed fat was washed out of the intestine with acetone and ether before its mucosa was removed for analysis.

In Table II are presented (1) the specific activities of phospholipide phosphorus, (2) the specific activities of acid-soluble phosphorus, and (3) relative specific activities, *i.e.* the ratio of 1:2. All dogs were sacrificed 5 to 6 hours after the introduction of fat into the lumen of the intestine, but at various intervals after the administration of the P^{32}. Thus P^{32} was administered at various intervals during the 6 hour period of fat absorption.

In most of the dogs the actual amount of fat absorbed was measured. In the remainder the presence of white lacteals from the pyloric sphincter up to the site of ligation was taken as evidence of active absorption.

TABLE II

Phospholipide Formation by Mucosa of Fat-Absorbing and Fasted Portions of Dog Small Intestine

Dog		Sacrificed after P^{32} injection*	Milky lacteals†	Fat administered‡	Fat absorbed	Specific activity of phospholipide P§		Specific activity of acid-soluble P§		Relative specific activity‖	
No.	Weight					Duodenal side	Ileal side	Duodenal side	Ileal side	Duodenal side	Ileal side
	kg.	*hrs.*		*gm.*	*gm.*	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$		
1	9.0	5.5		50 cc. cream, stomach tube		4.61	4.53	20.8	20.4	0.22	0.22
2	8.7	5.5		Same		6.87	6.57	27.5	23.4	0.25	0.28
3	10.9	6	+	50 cc. cream, duodenal side		31.2	32.2	81.6	99.0	0.38	0.33
4	10.9	6	−	Same		30.5	35.4	74.0	75.4	0.41	0.47
5	10.9	5	−	8.5 gm. corn oil, duodenal side	0	10.7	9.50	28.6	26.6	0.37	0.36
6	9.1	5	+	Same	8.3	16.0	11.7	30.8	25.5	0.52	0.46
7	14.1	5	+	"	5.1	5.21	4.27	13.4	14.4	0.39	0.30
8	8.7	5	+	"	8.4	7.69	7.06	32.0	29.0	0.24	0.24
9	11.0	5.25	−	8.5 gm. corn oil, ileal side		6.29	7.52	17.1	20.2	0.37	0.37
10	14.6	5.25	−	8.5 gm. corn oil, duodenal side	3.1	8.44	6.83	27.6	30.6	0.31	0.22
11	7.3	5	−	9 gm. corn oil fatty acids, ileal side	1	13.1	8.96	34.8	25.6	0.38	0.35
12	6.5	5	−	9 gm. corn oil fatty acids, duodenal side	5.1	11.7	10.1	35.0	28.0	0.33	0.36
13	9.6	5	+	Same	8.7	10.8	8.50	36.8	30.6	0.29	0.28
14	13.7	3	+	8.5 gm. corn oil, duodenal side	5.3	7.43	3.64	22.4	23.2	0.33	0.15
15	11.6	3	+	Same	7.2	6.84	7.38	25.2	26.6	0.27	0.28
16	16.1	3	+	"		4.18	4.81	21.1	20.1	0.20	0.23
17	12.2	1	+	"	1.7	4.96	3.29	28.0	30.4	0.18	0.11
18	15.2	1	+	"	1.2	2.82	4.80	35.2	35.7	0.080	0.13
19	11.2	0.25	+	"	4.9	1.39	1.21	29.6	26.0	0.047	0.047
20	9.8	0.25	+	"	8.0	0.582	0.988	10.7	18.6	0.054	0.053
21	12.7	0.25	+	"	8.2	0.379	1.79	11.6	30.2	0.033	0.059

* The P^{32} was injected intravenously. All dogs were sacrificed 5 to 6 hours after fat administration.

† Lacteals were white (+) or not white (−) in the duodenum at the end of the experiment.

‡ All fat was injected into the duodenal side of the ligated intestine unless otherwise indicated.

§ The specific activity is expressed as the per cent of the administered P^{32} per mg. of phosphorus.

‖ Relative specific activity is the ratio of specific activity of phospholipide P to that of acid-soluble P.

That ligation as carried out in this experiment does not interfere with phospholipide formation is evidenced by a comparison of the values for the relative specific activities of either portion of the ligated small intestine with those of the unligated small intestine at the 6 hour interval after the injection of the P^{32} (see Table I).

The results shown in Table II leave no doubt that the absorption of cream (an emulsified fat) or the absorption of corn oil or of its fatty acids does not increase the relative specific activity of the small intestine. This means that fat absorption in the dog is not accompanied by a significant increase in the rate of phospholipide formation by the small intestine.

Schmidt-Nielsen (7) has pointed out that phosphorylation of fat may be limited to the epithelial cells of the mucosa. Since these cells constitute but a small fraction of the entire mucosa, he argued that the increase in phospholipide formation induced in these cells by fat absorption would not be detectable when whole mucosa is analyzed. For this reason, in several dogs care was taken to remove the villi (as proved by subsequent histological examination) of the duodenal and ileal sides of the ligated small intestine. Here again no differences were observed in their relative specific activities when fat absorption was allowed to proceed in the duodenal side. It is therefore questionable whether, even in the epithelial cells, lipide phosphorylation is increased during fat absorption.

Lipide Phosphorylation in Small Intestine of Rat

Corn Oil—The first experiment was carried out on five groups of rats that had been fasted for 24 hours. All rats of each group were injected either subcutaneously or intramuscularly with radioactive phosphate, whereas only half of them were fed corn oil by stomach tube. The animals were sacrificed at various intervals from 0.25 to 9 hours after the administration of P^{32}. White intestinal lymphatics, evidence of fat absorption, were observed in all rats at the time they were sacrificed. In these animals the entire small intestine was used for determinations of phospholipide and acid-soluble phosphorus.

This experiment was designed to make possible a comparison of lipide phosphorylation in the small intestine of each fat-fed rat with its own control. Such comparisons are shown in each horizontal line of Table III.

The results obtained in this first experiment with the whole small intestine of the rat are in good agreement with those obtained with the mucosa of the dog. It should be pointed out, however, that previous investigations with P^{32} have led to quite contrary conclusions. Thus Schmidt-Nielsen (7), who used oleic acid and peanut oil, and Fries *et al.* (13), who used olive oil, observed a stimulation of phospholipide formation as measured with P^{32}. Hence in the next two experiments (Tables III and IV) a variety of oils was tested.

TABLE III

Phospholipide Formation by Whole Small Intestine of Fasted and Fat-Fed Rats*

Group	Rat No.	Hrs.		Rat weight		Fat administered	Fat absorbed	Phospholipide P^{31} per gm. dry weight		Specific activity of phospholipide P*		Specific activity of acid-soluble P*		Relative specific activity†	
		After fat administration	After P^{32} administration	Fat-fed	Control			Fat-fed	Control	Fat-fed	Control	Fat-fed	Control	Fat-fed	Control
				gm.	gm.	gm.	gm.	mg.	mg.						
A. Corn oil‡	1	9	9	320	326	0.85		4.17	3.80	0.352	0.248	0.498	0.457	0.71	0.54
	2	9	9	380	420	0.85	0.79	3.96	4.07	0.275	0.223	0.410	0.356	0.67	0.63
	3	9	9	250	470	0.85	0.81	3.68	3.40	0.372	0.235	0.502	0.394	0.74	0.60
B. " "‡	1	6	6	350	350	0.85		4.90	3.84	0.253	0.216	0.439	0.442	0.58	0.49
	2	6	6	330	320	0.85	0.85	3.91	4.50	0.228	0.209	0.342	0.426	0.67	0.49
	3	6	6	390	385	0.85	0.76	4.12	3.72	0.267	0.202	0.503	0.348	0.53	0.58
	4	6	6	420	440	0.85	0.83	3.84	4.00	0.192	0.193	0.428	0.380	0.45	0.51
	5	6	6	400	395	0.85	0.63	3.92	4.03	0.247	0.186	0.368	0.410	0.67	0.45
	6	6	6	345	330	0.85	0.83	4.57	4.12	0.245	0.218	0.462	0.430	0.53	0.51
C. " "‡	1	3	3	335	330	0.85	0.63	3.70	4.00	0.161	0.138	0.619	0.556	0.26	0.25
	2	3	3	320	310	0.85	0.68	3.86	3.86	0.185	0.137	0.685	0.678	0.27	0.20
	3	3	3	292	286	0.85		3.96	4.01	0.212	0.134	0.715	0.573	0.30	0.23
D. " "§	1	6	1	132	136	2.1		4.34	3.97	0.0592	0.0454	1.07	0.892	0.055	0.051
	2	6	1	150	140	2.1		4.60	4.56	0.131	0.0649	1.48	1.14	0.088	0.057
	3	6	1	145	120	2.1		4.27	4.69	0.130	0.000456	1.56	0.00532	0.083	0.086
	4	6	1	143	145	2.1		3.89	4.07	0.0838	0.0467	0.948	0.705	0.088	0.066
E. " "§	1	5.25	0.25	180	180	2.1		4.68	4.33	0.0228	0.0314	0.555	0.496	0.041	0.063
	2	5.25	0.25	180	180	2.1		4.44	3.98	0.0114	0.00633	0.452	0.224	0.025	0.028
	3	5.25	0.25	190	192	2.1		4.15	4.03	0.00540	0.00583	0.363	0.380	0.015	0.015
	4	5.25	0.25	162	160	2.1		4.37	4.52	0.0127	0.0113	0.502	0.534	0.025	0.021

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Peanut oil	1	5.25	0.25§	150	150	2.1		5.12	4.51	0.0181	0.00647	0.753	0.528	0.024	0.012
	2	5.25	0.25	136	140	2.1		4.82	4.58	0.0146	0.00876	0.810	0.591	0.018	0.015
	3	5.25	0.25	180	180	2.1		5.02	3.99	0.0241	0.0205	0.658	0.663	0.037	0.031
	4	5.25	0.25	160	160	2.1		4.87	4.52	0.0116	0.0115	0.685	0.638	0.017	0.018
	5	5.25	0.25	148	150	2.1		5.40	4.38	0.0278	0.0146	0.944	0.641	0.029	0.023
	6	5.25	0.25	148	145	2.1		5.19		0.0278	0.0422	0.895	0.716	0.031	0.059
	7	5.25	0.25	152	175	2.1		5.10	4.70	0.0180	0.0170	0.805	0.686	0.022	0.025
	8	5.25	0.25	145	146	2.1		5.49	5.15	0.0204	0.0373	0.813	0.664	0.025	0.056

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* The specific activity is expressed as the per cent of the administered P^{32} per mg. of phosphorus.

† Relative specific activity is the ratio of specific activity of phospholipide P to that of acid-soluble P.

‡ P^{32} injected subcutaneously; rats sacrificed with nembutal.

§ P^{32} injected intramuscularly; rats sacrificed by a blow on the head.

Comparison of Various Oils—The results obtained with peanut oil (Table III) are equally conclusive in failing to show a stimulation of lipide phosphorylation during fat absorption.

In the next experiment (Table IV), results obtained with cod liver oil, peanut oil, and corn oil were compared with those from a single control group. The animals were sacrificed 5 hours after the introduction of the fat into the gastrointestinal tract and 1 hour after P^{32} administration. Interestingly enough, a suggestive indication of an increase in lipide phosphorylation was observed for all three fats. But the increase observed in this experiment is too little to account for passage of the absorbed fat by way of phospholipide intermediates. It should be noted that the procedure used in this experiment was identical with that employed by Schmidt-

TABLE IV

Comparative Effects of Corn Oil, Peanut Oil, and Cod Liver Oil on Phospholipide Formation of Whole Small Intestine of Rat

Treatment	After fat administration*	After P^{32} administration	Rat weight	Phospholipide P^{31} per gm. dry weight†	Specific activity of phospholipide P†	Specific activity of acid-soluble P	Relative specific activity‡
	hrs.	*hr.*	*gm.*	*mg.*			
Corn oil, 2.1 gm.	5	1	218	4.35	0.0876	0.771	0.11
	5	1	170	4.42	0.148	1.39	0.11
	5	1	170	4.95	0.141	0.989	0.14
	5	1	156	4.33	0.244	1.37	0.18
	5	1	163	4.38	0.181	1.28	0.14
Peanut oil, 2.1 gm.	5	1	218	4.65	0.0768	0.760	0.10
	5	1	167	4.23	0.104	0.751	0.14
	5	1	166	4.71	0.184	1.23	0.15
	5	1	155	4.51	0.126	1.12	0.11
	5	1	146	4.95	0.0998	1.03	0.10
Cod liver oil, 2.1 gm.	5	1	190	4.93	0.205	0.972	0.21
	5	1	184	4.49	0.203	1.15	0.18
	5	1	166	4.65	0.144	0.985	0.15
	5	1	156	4.42	0.188	1.29	0.15
Controls, no fat		1	192	3.75	0.0701	0.785	0.089
		1	185	4.00	0.0525	0.958	0.055
		1	168	3.97	0.0854	1.13	0.076
		1	162	4.03	0.0850	1.02	0.083
		1	134	2.82	0.114	1.15	0.099

* Lacteals appeared white at the time of sacrifice.

† The specific activity is expressed as the per cent of the administered P^{32} per mg. of phosphorus.

‡ Relative specific activity is the ratio of specific activity of phospholipide P to that of acid-soluble P.

Nielsen (7), who under these conditions observed a 4-fold increase in the specific activities of phospholipide phosphorus in the small intestine of the rat absorbing peanut oil.

Effect of Age—The results shown in Table V offer no evidence that young rats weighing from 80 to 122 gm. differ in their response to ingested fat from older rats.

As noted above, the results obtained in the present investigation differ from those obtained by other investigators in the effect of fat absorption upon lipide phosphorylation in the small intestine of the rat. In attempting to explain this difference, attention was directed to the possibility that the experimental procedure employed by the various investigators was not the same. In the present investigation we have ruled out the possibility that factors such as the oil used, the age of the rat, and the interval after fat and P^{32} administration could explain the discrepancy. Artom and Cornatzer (14) have recently pointed out that "the supply of choline (or choline precursors) may represent a limiting factor for the formation of phospholipides during the absorption of fat from the intestine." It is unlikely that the 24 hour fast, to which all rats were subjected in this investigation, could have created a choline deficiency. Since Artom and Cor-

TABLE V

Effect of Age on Phospholipide Formation of Whole Small Intestine of Fasted and Fat-Fed Rats

All rats were sacrificed 5 hours after fat feeding and 1 hour after P^{32} administration.

Weight range	Phospholipide P^{31} per gm. dry weight			Specific activity of phospholipide P*			Specific activity of acid-soluble P*			Relative specific activity†		
	Controls	Corn oil	Cod liver oil	Controls	Corn oil	Cod liver oil	Controls	Corn oil	Cod liver oil	Controls	Corn oil	Cod liver
gm.	*mg.*	*mg.*	*mg.*									
80–97	4.30	4.74	4.80	0.0893	0.181	0.256	1.11	1.52	2.10	0.081	0.12	0.12
			4.42			0.192			0.158			0.12
112–122	2.79	4.63	4.25	0.0954	0.200	0.168	0.857	1.08	1.65	0.11	0.19	0.10
	4.24	4.75	4.45	0.0708	0.121	0.151	0.527	0.955	1.23	0.13	0.13	0.12
	4.47	4.18		0.0716	0.146		1.07	1.12		0.07	0.13	
210–245	3.93	4.96	4.12	0.0517	0.0590	0.159	0.803	0.966	1.19	0.06	0.06	0.13
	3.83	4.16		0.0386	0.0637		1.06	0.953		0.04	0.07	
252–260	4.12	4.75	4.33	0.0500	0.168	0.114	0.910	1.03	1.06	0.06	0.16	0.11
			3.90			0.104			0.667			0.16
290–300	4.02	4.40	4.15	0.0870	0.0864	0.135	0.835	0.867	0.123	0.10	0.10	0.11

* The specific activity is expressed as the per cent of the administered P^{32} per mg. of phosphorus.

† Relative specific activity is the ratio of specific activity of phospholipide P to that of acid-soluble P.

natzer observed in choline-deficient rats that fat absorption occurred without an increase in phospholipide formation by the small intestine, their findings provide additional support for the conclusion offered here that phospholipides are not obligatory participants in fat absorption.

DISCUSSION

According to the concept first expressed by Sinclair (3), phospholipides are intermediates in the synthesis of neutral fat from fatty acids within the intestinal wall. Bloor in 1943 (15) described the mechanism as follows: "As soon as the fatty acids are absorbed into the epithelial cells, molecules of phospholipide react with the free fatty acids (or soaps) to form neutral fat;[2] immediately, however, the residual phosphoric acid-base complex[3] unites with the newly absorbed fatty acids and glycerol to form phospholipide."

The evidence dealing with the question whether phospholipides form an obligatory step in fat absorption may be grouped according to the following methods of study: (1) labeled fatty acids, (2) inhibitors and accelerators of lipide phosphorylation, (3) lymph phospholipides, and (4) radioactive phosphorus. Many of these investigations have been reviewed by Verzár and McDougall (17), Bloor (15), and Frazer (18).

That fatty acids present in the intestinal lumen are incorporated into the lipide fractions of the mucosa has been amply demonstrated with such labeling agents as elaidic acid, iodized fatty acids, and conjugated fatty acids. The significance of their appearance in mucosal phospholipides, from a qualitative point of view, has been dealt with in the "Introduction." Moreover, Barnes *et al.* were impressed by the fact that at the height of fat absorption, when neutral fat in the mucosa contained large amounts of administered labeled fatty acid, only small amounts of their labeled fatty acids appeared in the phospholipide fraction of the small intestine (6). They therefore concluded that phosphorylation is not an essential part of fat transport through the intestine.

As evidence favoring phospholipides as intermediates in fat absorption, Verzár and his associates offered their findings that iodoacetic acid, phlorhizin, and adrenalectomy interfered with fat absorption as well as with phosphorylations in the intestine (17). Klinghoffer has shown that iodoacetic acid induces pathological changes in the intestine, so that it is unnecessary to invoke an interference in phosphorylation to account for a depressed fat absorption in the animal treated with this drug (19). The work of Barnes *et al.* leaves no doubt that the fatty acid-glycerol ester linkage can be formed in the adrenalectomized rat (20), whereas Stillman *et al.* offered direct proof that phosphorylation of fat, as measured by the incorporation of administered radiophosphorus into the phospholipide molecule, is not interfered with in the small intestine of the adrenalectomized rat (21).

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[2] The other possibility, namely that glycerol choline serves as a stable skeleton to carry fatty acids, can, for obvious reasons, not be tested with the radioactive isotope of phosphorus. This possibility is being investigated with C^{14}-labeled palmitic acid.

[3] Since Riley (16) found that administered phosphoryl choline phosphorus is rapidly converted to inorganic phosphorus, it should not be inferred that even the phosphoric acid-nitrogen base complex enters and leaves the phospholipide molecule as a stable unit.

Verzár and Laszt claimed that fat absorption can be accelerated by the addition of either glycerol and phosphate or glycerophosphate (22). This finding has been confirmed by Cera and Bellini (23). The Verzár school interprets these experiments as meaning that during its absorption fat is transformed in the intestinal mucosa to phospholipide, the latter being intermediate in the synthesis of neutral fat in the epithelial cells (17).

Many workers have shown that the phospholipide content of intestinal lymph or thoracic duct lymph is increased during fat absorption. This has been confirmed by Flock *et al.* (24), as well as in this laboratory. It has been pointed out that phospholipide fatty acids contained in lymph account for very little of the fat absorbed, much the largest portion of the extra fatty acids contained in lymph being present as triglycerides. This argument, however, need not invalidate the concept of lipide phosphorylation in fat absorption because (1) the amount of extra phospholipides carried by portal blood during fat absorption has not been satisfactorily measured and (2) it is conceivable that, if phospholipides are formed as intermediates in neutral fat synthesis, they do not escape from the intestinal wall.

In the rat injected with P^{32} an increase in the specific activity of the intestinal phospholipide phosphorus during fat absorption has frequently been reported (7, 13). The extensive observations reported here on rat and dog, however, do not confirm the earlier observations. The more recent findings of Schmidt-Nielsen (7) deserve particular mention. Although he found an increase in the specific activity of phospholipide phosphorus of the rat's small intestine during fat absorption, he calls attention to the fact that the increase is insufficient to account for the conversion of the absorbed fat to phospholipide, unless the phosphorylation process is limited to the epithelial cells. But even this limitation would appear inadequate to explain our failure to observe any increase whatsoever in the relative specific activities of the phospholipides of the villi in the small intestine of the fat-absorbing dog.

The inconclusive nature of the above earlier evidence offered in support of phosphorylation, coupled with the more definite finding of the present investigation, namely that fat absorption does not stimulate the rate of phospholipide turnover in the whole small intestine or in its mucosa, leads us to conclude that fat can pass through the intestinal wall without involving phospholipide as an obligatory intermediate.

SUMMARY

1. A discussion is presented on the use of relative specific activities of phospholipide phosphorus for comparing phospholipide turnover under different experimental conditions.

2. In the dog, neither the amount nor the tunover of the phospholipide of the mucosa or the villi of the small intestine is affected by the absorption of cream, corn oil, or corn oil fatty acids.

3. In the rat, such increases in the relative specific activities of phospholipide phosphorus of the small intestine as may occur during fat absorption are too small to account for all of the absorbed fat having passed through a phospholipide stage.

4. The findings presented here fail to support the hypothesis that phospholipides are obligatory intermediates in the passage of absorbed fat through the intestinal wall.

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HOMOSERINE AS A PRECURSOR OF THREONINE AND METHIONINE IN NEUROSPORA

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Previous work on the synthesis of methionine in *Neurospora* (1) has established that the sulfur atom is derived from cysteine by way of the following series of gene-determined steps:

Cysteine → cystathionine → homocysteine → methionine

The same pathway can account for the formation of cysteine from methionine in animals (2). The key intermediate in these transformations is the asymmetrical thioether cystathionine; this compound mediates the transfer of the sulfur atom from the 3-carbon chain of cysteine to the 4-carbon chain of homocysteine in *Neurospora* and vice versa in animals.

The experiments of Binkley and du Vigneaud (2) suggest that homocysteine and serine condense in the rat liver to form cystathionine which cleaves to give cysteine. Binkley (3) has shown that cleavage of cystathionine by liver is accompanied by the disappearance of one high energy phosphate bond; it was suggested that the product of the cleavage reaction in addition to cysteine is the phosphate ester of α-amino-γ-hydroxybutyric acid (phosphohomoserine).

We have recently obtained evidence that homoserine is the precursor of the 4-carbon chain of cystathionine (and therefore of the carbon skeleton of methionine) in *Neurospora*. At the same time, an unexpected and not yet fully understood relationship between the biological syntheses of methionine and of threonine was discovered.

Materials and Methods

Mutant Strain—This paper deals with strain 51504, a mutant of *Neurospora crassa* obtained at the Stanford laboratories by ultraviolet irradiation of asexual spores. Crosses of this mutant with strains normal with regard to amino acid requirements were made and ascospores were dissected and cultured. Of these asci, 89, of which at least one member of each spore pair germinated, were analyzed for the mutant character. All 89 asci showed two pairs of mutant spores and two pairs of normal spores. These are the results to be expected if the mutant character differs from the normal by a single gene. Tests carried out on cultures derived from several complete asci showed that recovered strains have the same growth requirements, qualitatively and quantitatively, as the parental strains.

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Linkage studies with morphological and biochemical mutant markers showed that the gene conditioning the mutant character of strain 51504 is located on the first chromosome on the arm opposite the sex gene, about 25 crossover units from the centromere. For techniques of crossing and ascus dissection and testing, see Beadle and Tatum (4).

Growth of the mold was measured as the dry weight of mycelium produced after 72 hours incubation at 25°. Cultures were grown in 20 ml. of medium in 125 ml. Erlenmeyer flasks. The basal medium and the techniques for inoculation, drying the pads, and keeping stock cultures have been described elsewhere (5).

Compounds—DL-Homoserine was synthesized in the following manner. α-Acetobutyrolactone, prepared by the procedure of Knunyantz *et al.* (6), was converted to the phenylhydrazone of α-ketobutyrolactone by the method of Harradence and Lions (7). The reduction of the phenylhydrazone with tin and HCl to yield homoserine has been reported by Feofilaktov and Onishchenko (8). It was found, however, that the reduction goes more smoothly with zinc and methanolic hydrogen chloride, according to the procedure used by Feofilaktov for the preparation of other amino acids (9). 105 gm. of phenylhydrazone and 100 gm. of zinc dust were suspended in 1 liter of 95 per cent ethanol. The mixture was placed in an ice bath and 500 ml. of 25 per cent hydrogen chloride in methanol were added with stirring over a period of 6 hours. The unchanged zinc was filtered off and the alcohol and excess HCl removed from the filtrate by concentration under reduced pressure. The residue was dissolved in water and treated successively with lead carbonate, silver carbonate, hydrogen sulfide, and nuchar. The colorless solution was evaporated to dryness over a water bath. The residue was crystallized by dissolving in water and adding 95 per cent ethanol. The yield after recrystallization was 15.2 gm., m.p. 180–181°.

In the preparation of D-methionine, DL-methionine was formylated according to the method of du Vigneaud *et al.* (10) for DL-cystine. 10 gm. of DL-methionine were dissolved in 120 ml. of 88 per cent formic acid. The temperature was brought to 59–61° and maintained there by the slow addition of 42 ml. of acetic anhydride. When the mixture was cool, 21 ml. of water were added and the clear solution was concentrated under reduced pressure. Upon crystallization of the residue from ethyl acetate, there were obtained 9.1 gm. of formyl-DL-methionine, m.p. 100–101°. The resolution of formyl-DL-methionine and the recovery of D-methionine were carried out according to the procedure of Windus and Marvel (11).

L-Cystathionine was isolated from strain *mc-2* (H98) of *Neurospora crassa*, s described by Horowitz (1). DL-Homocysteine thiolactone hydrochloride as prepared by the method of Riegel and du Vigneaud (12). D- and -threonine were kindly made available by Dr. R. T. Major of the Merck esearch Laboratories.

RESULTS AND DISCUSSION

Strain 51504 was originally classified as a *threonineless* mutant, since hreonine was the only amino acid tested upon which it showed any growth, lthough even here growth was scant. The following twenty-five amino cids, tested singly in concentrations of 1 mg. per 20 ml. of medium, do not upport the growth of this strain: DL-threonine (slight growth), DL-isoleucine, DL-methionine, L-histidine, DL-valine, L-lysine, L-tryptophan, DL-leucine, glycine, DL-alanine, DL-aspartic acid, L-proline, L-arginine, hydroxy-L-proline, L-glutamic acid, DL-serine, L-tyrosine, L-cystine, L-cysteine, DL-norleucine, DL-phenylalanine, DL-norvaline, DL-α-aminobutyric acid, DL-citrulline, and DL-ornithine.

TABLE I

Effect of Various Supplements on Growth of Strain 51504

The indicated amounts of supplement were added to 20 ml. of basal medium. Growth was measured as the dry weight of mold produced after 72 hours at 25°.

Supplement	Growth
	mg.
None	0
5 mg. hydrolyzed casein	0
5 “ “ “ + 2 mg. DL-threonine	45.0
1 “ DL-threonine	2.0
1 “ “ + 1 mg. DL-methionine	43.0
1 “ “ + 1 “ DL-homocysteine thiolactone hydrochloride	27.5
1 “ “ + 1 “ L-cystathionine	34.0
1 “ “ + 1 “ L-cysteine hydrochloride	2.0
1 “ “ + 1 “ L-cystine	2.0
1 “ “ + 1 “ choline chloride	13.0
1 “ L-threonine + 1 “ L-methionine	33.0
1 “ D-threonine + 1 “ “	0

Further study of the growth requirements of the mutant showed that it responds to threonine plus some factor or factors present in casein hydrolysate, although no response was obtained with casein hydrolysate alone (Table I). Experiments were carried out to discover whether any of the amino acids mentioned above would replace the factor in casein hydrolysate. Each of the twenty-five amino acids was tested with threonine in 1 mg. amounts. Methionine was found to be the only one which supports growth in the presence of threonine.

Attempts to replace the threonine portion of the requirement were carried out by supplying each of the twenty-five amino acids in the presence of methionine. None of them was found to replace threonine. Tests with L- and D-threonine showed that only the natural form is active for the mutant (Table I). This provides further support of the idea that the D-amino acid oxidase plays an essential part in the inversion of the unnatural enantiomorphs of the amino acids, since it has been shown that the D-amino acid oxidase of *Neurospora* does not attack D-threonine (13).

Further tests were made to determine whether known precursors of methionine are able to fulfil the methionine requirement of the mutant. The results (Table I) show that cystathionine and homocysteine (as the thiolactone), but not cysteine, will promote growth of this strain when supplied together with threonine. Choline shows some activity, presumably by virtue of a sparing action on methionine. It is inferred from these results that the methylation of homocysteine and the cleavage of cystathionine to yield homocysteine proceed normally in the mutant, but that the synthesis of cystathionine from cysteine is blocked. In regard to blocking methionine synthesis, strain 51504 thus resembles strain *me-3* described previously (1); it differs from the latter in that the block to cystathionine synthesis in mutant 51504 at the same time abolishes threonine synthesis.

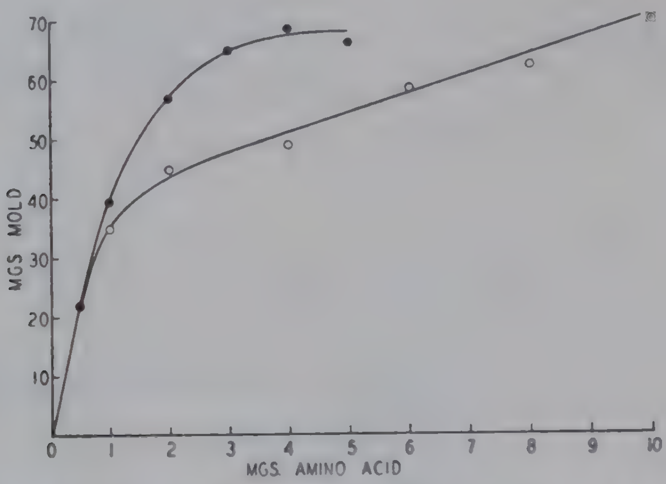
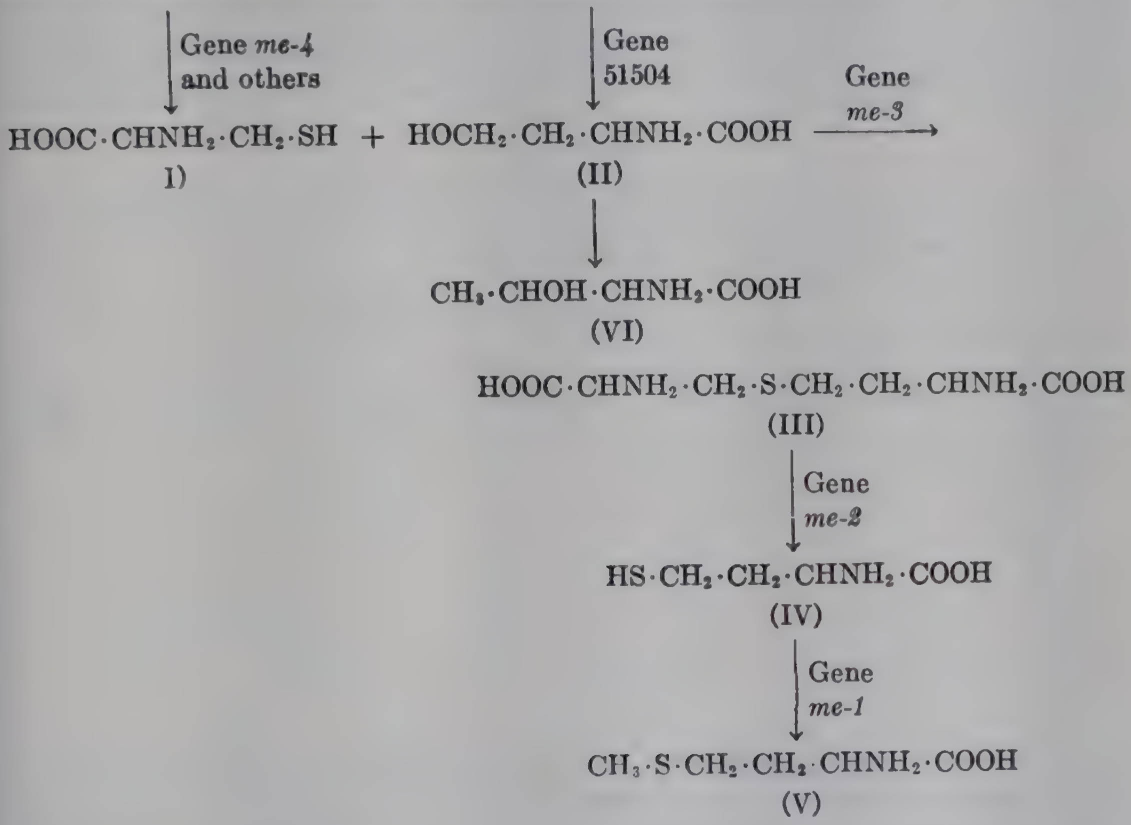


Fig. 1. Growth of strain 51504 on DL-homoserine (●) and on a mixture containing equal parts of DL-methionine and DL-threonine (○).

In explanation of the nutritional requirements of strain 51504, the possibility immediately suggests itself that cystathionine and threonine have a common precursor whose synthesis is blocked in the mutant. Strong evidence for this hypothesis would be obtained if a substance could be found which, when supplied to the organism, satisfies both the methionine and the threonine requirement. The tests described above showed that none of the common amino acids can replace both threonine and methionine in promoting the growth of the mutant. It was therefore decided to test homoserine; although there was no basis for assuming it to be biochemically related to threonine, homoserine suggested itself as a precursor of cystathionine by analogy with the serine-homocysteine reaction in the rat liver (2, 3).

DL-Homoserine was synthesized by the method described above and, when tested on strain 51504, was found to be active. It appears, therefore, that homoserine gives rise to both methionine and threonine in *Neurospora*.



The activity of DL-homoserine for the mold is equal to or better than that of a mixture of DL-threonine and DL-methionine (Fig. 1), a fact which adds weight to the idea that homoserine is a normal biological precursor of threonine and methionine. In the accompanying diagram this conclusion is incorporated into the previously arrived at scheme of methionine synthesis in *Neurospora* (1); the genes known to control various stages of the process are indicated. Cysteine (I) condenses with homoserine (II), giving rise to cystathionine (III); cystathionine is cleaved to homocysteine (IV), which is methylated to yield methionine (V); in a separate reaction, or series of reactions, homoserine gives rise to threonine (VI).

Although the mechanism of the conversion of homoserine to threonine is not known, an obvious possibility is that of a shift of the hydroxyl group to the β position. The first step in this rearrangement might be a dehydration of homoserine to the β,γ-unsaturated amino acid, analogous to the reaction suggested for serine and threonine by Chargaff and Sprinson (14). Rehydration to threonine might then take place. Still other mechanisms suggest themselves; for example, it is conceivable that homoserine acts as a specific amino, or even hydroxyl, donor to the immediate precursor of threonine.

Methionine Inhibition—In an experiment designed to determine the minimum amounts of threonine and methionine required for optimal growth of the mutant, it was discovered that an excess of methionine inhibits growth. The inhibition appears to involve a competition between methionine and threonine, since it is relieved by increasing the threonine concentration, as shown in Table II. When the effects of L- and DL-methionine were compared, the surprising result was obtained that L-methionine is twice as inhibitory as the racemic mixture, leading to the conclusion that the inhibitory action is confined to the natural isomer. Confirmation of this result was obtained in experiments in which the mutant was grown with threonine plus varying amounts of D-methionine. As is shown in Table III, both enantiomorphs support the growth of the strain, but only L-methionine is inhibitory in the concentration range tested. To explain this result it seems necessary to assume that D-methionine is converted to L-methionine at a rate sufficient to provide for the growth of the mutant, but not at a rate sufficient to give rise to an inhibitory concentration of the natural enantiomorph.

TABLE II

Effect of Varying Threonine and Methionine Concentrations on Growth of Strain 51504

The values represent growth of the mold in mg.

DL-Threonine	DL-Methionine, mg. per 20 ml.				
	0	0.1	1.0	5.0	10
mg. per 20 ml.					
0	0	0	0	0	0
0.1	0	0	0	0	0
0.5	0	32	1	0	0
1.0	1	40	47	0	0
2.0	2	47	56	1	0
5.0	2	58	62	64	2
10.0	3	59	59	53	50

The critical molar ratio of L-methionine to L-threonine, above which inhibition sets in, is approximately 0.8. Inhibition is complete when the ratio exceeds 2. The exact ratios vary with the age of the culture used in testing, older cultures being somewhat more sensitive to methionine inhibition than fresh ones. Inhibition by methionine is also observed when the mutant is grown on homoserine (Table III).

The growth of the wild type *Neurospora* is not inhibited by DL-methionine in a concentration of 10 mg. per 20 ml., or by various mixtures of threonine and methionine. The effect appears to be specifically related to strain 51504 and is similar in this respect to the inhibition of the growth of lysine-requiring mutants of *Neurospora* by L-arginine, described by Doermann (15). Tests with other amino acids have shown that the inhibitory action is not confined to L-methionine. DL-Norvaline, L-tyrosine, and DL-α-aminobutyric acid were all found to suppress growth of strain 51504 when added in 1 mg. amounts to a medium containing optimal concentrations of threonine and methionine; it is probable that still other amino acids will be found to inhibit the growth of this strain. The failure of the mutant to grow in medium supplemented with casein hydrolysate (see above) is thus explained as resulting from the inhibitory effects of various amino acids.

TABLE III

Inhibition of Growth by L-Methionine

The values represent growth of the mold in mg.

Supplement	Methionine configuration		
	DL-	L-	D-
1 mg. DL-threonine + 0.5 mg. methionine	44.0	48.5	43.0
1 “ “ + 1.0 “ “	46.0	20.0	40.0
1 “ “ + 1.5 “ “	42.0	0.5	41.5
1 “ “ + 2.0 “ “	21.0	0	40.5
1 “ “ + 3.0 “ “	0	0	41.5
1 “ “ + 5.0 “ “			39.0
1 “ DL-homoserine + 1 “ “		54.0	
1 “ “ + 2 “ “		10.0	
1 “ “ + 5 “ “		1.0	

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SUMMARY

A monogenic, radiation-induced mutant of *Neurospora* which requires methionine and threonine for growth is described. The methionine requirement can be satisfied by homocysteine or cystathionine, but not by cysteine. Both the methionine and the threonine requirments can be satisfied by homoserine. These results indicate that homoserine functions as a pre-

cursor of threonine in the organism and also gives rise to the 4-carbon chain of cystathionine.

The mutant utilizes L-threonine but not D-threonine for growth, whereas both enantiomorphs of methionine are active. Growth of the strain is inhibited by excess L-methionine; the inhibition can be reversed by increasing the threonine concentration. D-Methionine is not toxic to the mutant in any concentration tested.

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THE ENERGY METABOLISM OF CLOSTRIDIUM KLUYVERI AND THE SYNTHESIS OF FATTY ACIDS

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Clostridium kluyveri is an anaerobic bacterium which was shown several years ago to convert ethanol to caproic acid (1–3). This unusual process could not be adequately studied at that time because of difficulties in growing the organism. These difficulties have recently been overcome and it is now possible to grow the bacterium in any desired quantity in a completely synthetic medium with relative ease (4). Nutritional studies have shown that *Clostridium kluyveri* is unable to attack substrates like glucose and pyruvate which are commonly fermented by other anaerobic bacteria, but it obtains energy by the simultaneous decomposition of ethanol and a salt of one of the lower fatty acids, which may be acetate, propionate, or butyrate. It is the purpose of this paper to describe the over-all chemical reactions that occur during the transformation of these substrates. It will be shown that the main fermentation products are acetic, *n*-butyric, *n*-valeric, *n*-caproic, and *n*-heptanoic acids and hydrogen. Also some evidence concerning the mechanism of formation of these fatty acids will be presented.

Methods

Culture Medium—All experiments were done with growing cultures of *Clostridium kluyveri*, strain K.1. The following Medium 1 was used: ethanol 0.1 to 2.0 gm., sodium salt of a fatty acid 0.1 to 1.5 gm., KH_2PO_4-Na_2HPO_4 buffer (1 M, pH 7) 2.5 ml., $(NH_4)_2SO_4$ 50 mg., $MgSO_4 \cdot 7H_2O$ 20 mg., $CaSO_4 \cdot 2H_2O$ 1 mg., $FeSO_4 \cdot 7H_2O$ 0.5 mg., $MnSO_4 \cdot 4H_2O$ 0.25 mg., $NaMoO_4 \cdot 2H_2O$ 0.25 mg., Difco yeast extract 75 mg., $Na_2S \cdot 9H_2O$ 20 mg., Na_2CO_3 10 mg., and glass-distilled water 100 ml. In small scale experiments the sulfide was usually replaced by 50 mg. of sodium thioglycolate. All cultures were incubated under strictly anaerobic conditions at 35°.

Chemical Methods—Gases were collected over mercury and analyzed in a Fisher gas analysis apparatus. Dissolved carbon dioxide was estimated by the manometric method described by Peters and Van Slyke (5), alcohol by a semimicro modification of the method of Northrop *et al.* (6), following distillation, and total volatile acids by steam distillation and titration.

The separation of fatty acids in mixtures was accomplished by distillation in an efficient fractionating column at reduced pressures. The individual acids were identified by determining their physical properties, including

Duclaux distillation constants, refractive indices, melting and boiling points of the free acids, and melting points of the acid amides. The identification of the higher fatty acids will be considered in detail in connection with the individual experiments.

For the estimation of acetic, butyric, and caproic acids in a mixture, the Duclaux distillation method was used (7). Tests on known mixtures showed that the error in the estimation of an individual acid is usually within ±5 per cent, provided the acid constitutes more than one-fifth of the total acid in the sample.

The quantitative analysis of mixtures of fatty acids containing acetic, propionic, *n*-butyric, *n*-valeric, *n*-caproic, and *n*-heptanoic acids will be discussed in connection with the ethanol-propionate fermentation.

EXPERIMENTAL

Ethanol-Acetate Fermentation—A large scale fermentation was carried out to provide enough material to permit positive identification of the products. For this purpose a 12 liter culture of Medium 1 containing 0.65 per cent ethanol and 0.5 per cent sodium acetate was incubated for 12 days until growth had ceased and was then analyzed.

The residual neutral volatile compounds were collected by distillation and tested for aldehydes and ketones with 2,4-dinitrophenylhydrazine with negative results. The alcohol was then oxidized with acid dichromate to the corresponding fatty acid, which was demonstrated to be pure acetic acid by the Duclaux distillation method. It may be concluded that ethanol was the only alcohol present and no other neutral volatile compounds were formed during the fermentation.

About 35 ml. of mixed fatty acids were separated and subjected to fractional distillation. Six fractions were collected as shown in Table I. Fraction A was identified as acetic acid by Duclaux distillation and by the equivalent weight. Fraction B was found by Duclaux distillation to consist mainly of acetic acid contaminated with a small quantity of a higher acid, probably butyric acid. Fractions C and D were identified as *n*-butyric acid. The Duclaux distillation values for these fractions are identical with those for synthetic *n*-butyric acid and the equivalent weight is approximately correct. Both the melting and boiling points correspond to those for *n*-butyric acid; the values are very different from those of isobutyric acid, which has a melting point of −47° and a boiling point of 154.4°. Fractions E and F were identified as caproic acid by Duclaux distillation and the equivalent weight determination. The boiling, melting, and freezing points identify the caproic acid as the normal isomer. The boiling points of other caproic acid isomers lie between 183.5–199° and the melting points are between −1.5° and −42°.

Additional evidence concerning the identity of the caproic acid isomer was obtained by determining its infra-red absorption spectrum.[1] Samples of the acid were dissolved in both carbon disulfide and carbon tetrachloride and its spectrum was compared in the region from 2 to 15 mμ with that of a sample of *n*-caproic acid obtained from the Carbide and Carbon Chemicals Corporation, and described as having a purity of better than 98 per cent. The two samples had identical spectra, except that the bands at 6.59 and 13.13 mμ were stronger in the bacterial preparation. This indicates that the latter is mostly *n*-caproic acid, containing a small amount of some impurity which is common to it and to the reference sample. The nature or source of this impurity was not determined.

TABLE I

Physical Properties of Fatty Acid Fractions from Ethanol-Acetate Fermentation

Fraction	Distillation data			Refractive index, 22°	Equivalent weight	B.p.	M.p.
	Pressure	Temperature	Amount				
	mm. Hg	°C.	*ml.*		*gm.*	°C.	°C.
A	90	52–72	1.65	1.3718	60.2		
B	90	75–100	0.60	1.3801			
C	18	72–79	10.00	1.3981	87.7	162.8	−7.2 to −8
D	18	79–90	0.95	1.3981			
E	5	80–99	22.10	1.4164	116.1	203.6	−2.0 (−9)*
F†			2.70				

* Freezing point, which is normally lower than the melting point with *n*-caproic acid.

† Residue from Fraction E.

From the above results one may conclude that the volatile acids present at the end of an ethanol-acetate fermentation consist mainly if not entirely of acetic, *n*-butyric, and *n*-caproic acids. Quantitative data on this fermentation are given in Table II. It is evident that butyrate, caproate, and hydrogen account for nearly all the ethanol and acetate consumed.

Ethanol-Propionate Fermentation—A culture consisting of 5.5 liters of Medium 1, containing 1.5 per cent sodium propionate and 2 per cent ethanol, was incubated for 18 days until growth ceased and was then analyzed.

The neutral volatile fraction of this fermentation consisted of ethanol containing a small amount of a higher alcohol which was not identified. No aldehydes or ketones could be detected.

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[1] The infra-red spectra were determined by Dr. R. R. Brattain of the Shell Development Company. The authors wish to express their appreciation to Dr. Brattain and to Dr. Otto Beeck for making it possible to obtain this information.

About 60 ml. of mixed volatile acids were recovered from the fermented medium. A small sample of the mixture was subjected to an azeotropic distillation (8), with benzene, toluene, and xylene, successively, in order to learn its approximate composition. Control experiments with a known mixture of fatty acids showed that the benzene distillate contains acetic acid and sometimes a small quantity of propionic acid, the toluene distillate contains most of the propionic and butyric acids, the xylene fraction contains valeric and caproic acids, and the residue from the xylene distillation contains heptanoic and higher acids. The approximate composition of each fraction was determined by Duclaux distillation. The distillation data indicated the presence of acetic, propionic, butyric, valeric, and caproic acids in considerable amounts and also a small amount of a higher acid. To obtain more conclusive evidence as to the identity of the individual acids, the mixed fatty acids were subjected to a fractional distillation. Eight fractions were collected; these are listed in Table III along with some of their physical properties.

TABLE II

Ethanol-Acetate Fermentation

		mM per 100 ml.
Substrates* decomposed	Ethanol	4.55
	Acetate	2.18
Products formed	Butyrate	1.04
	Caproate	1.46
	Hydrogen†	1.11
Carbon recovery, %		96
Oxidation-reduction index		1.00

* Initial and final ethanol concentration, 8.37 and 3.82 mM per 100 ml., respectively; initial and final acetate concentration, 3.57 and 1.38 mM per 100 ml., respectively.

† Determined in a parallel fermentation of smaller volume.

Fraction A undoubtedly consists of a mixture of water, acetic acid, and propionic acid. This is indicated by the distillation temperature range and by Duclaux distillation data, which correspond to a 3:2 mixture of acetic and propionic acids. Fraction B consists of almost pure propionic acid. This conclusion is supported by the data on the distillation temperature range, by the Duclaux distillation data, and by the refractive index: observed 1.3858, propionic acid 1.3873. The same types of evidence and the characteristic odor indicated that the small Fraction C is a mixture of about equal parts of propionic and butyric acids. Fraction D is almost pure butyric acid. The identity of the butyric acid isomer was not investigated in detail. Since butyric acid is undoubtedly formed from acetate, as in the ethanol-acetate fermentation, it seems safe to assume that here we are also dealing with the normal isomer.

Fractions E to H were examined with special care to establish the identity of the isomers as well as the number of carbon atoms. Fractions E and F, comprising more than half the total acid, appear to consist of a single compound, *n*-valeric acid. The Duclaux data, equivalent weight determination, and distillation temperature range identify the compound as a valeric acid. It is possible to distinguish the normal from the other three isomers by the following properties: b.p. 184.5–185.5°, *n*-valeric acid 186°, other isomers 163.8–176.5°; m.p. −35.0° to −36.5°, *n*-valeric acid −34.5°, other isomers −80°, −30°, and 35.5°; m.p. of the acid amide 105.5°, *n*-valeramide 106°, amides of other isomers 112–135°. α-Methylbutyric acid was completely eliminated as a possible constituent of Fractions E and F by a determination of the number of terminal methyl groups by the method of Ginger (9). The yield of acetic acid was 0.82 mole per mole of fatty acid. Under the same conditions, α-methylbutyric acid gave 1.48 moles of acetic acid and *n*-valeric acid 0.82 mole per mole.

TABLE III

Physical Properties of Fatty Acid Fractions from Ethanol-Propionate Fermentation

Fraction	Distillation data			Equivalent weight	B.p.	M.p.	M.p. of amide
	Pressure	Temperature	Amount				
	mm. Hg	°*C.*	*ml.*	*gm.*	°*C.*	°*C.*	°*C.*
A	88	47– 81	12.0				
B	88	81–112	8.6				
C	19	63– 73	1.4				
D	12	64– 78	1.7				
E	2–3	59– 66	13.0	101.8	184.5	−36.5	106*
F	2–3	66– 82	18.2	102.1	185.5	−35.0	106*
G	2–3	82– 91	3.4	116.4	204.0	−2.0 (−9.5)†	100*
H‡			1.4	130.4	221.0	−6.0	96*

* Mixed melting points with amides of normal isomers.

† Freezing point.

‡ Distillation residue.

There was a sharp rise in the distillation temperature between Fractions F and G. Most of Fraction F distilled below 77°, while most of Fraction G distilled at 90°. All the data presented in Table III and the Duclaux distillation data indicate Fraction G to be *n*-caproic acid. Fraction H was the residue left after distillation of the preceding fractions. The physical properties of the acid and its amide indicate it to be *n*-heptanoic acid. The acids of both Fractions G and H were shown to possess only a single terminal methyl group by the method of Ginger (9).

On the basis of the above observations it may be concluded that the fatty acids formed in the ethanol-propionate fermentation are acetic, *n*-butyric, *n*-valeric, *n*-caproic, and *n*-heptanoic acids. The only other product formed in a significant amount is hydrogen.

An accurate quantitative estimation of all the fatty acids in a mixture containing six components cannot be achieved by any method so far developed. However, the approximate quantities of the individual acids could be calculated from the total volatile acidity, determined by direct steam distillation of the medium, and a knowledge of the fraction of the total volatile acid corresponding to each component. The latter values could be estimated from the fractional distillation data presented in Table III and from data on the composition of each fraction, obtained by Duclaux distillation. The results, calculated in this manner, are presented in Table IV. The carbon recovery (93 per cent) and oxidation-reduction index (1.18) are such as to indicate that the data are reasonably reliable.

Table IV

Ethanol-Propionate Fermentation

		mM per 100 ml.
Substrates* decomposed	Ethanol	12.2
	Propionate	10.0
Products	Acetate	2.9
	n-Butyrate	0.65
	n-Valerate	7.2
	n-Caproate	0.7
	n-Heptanoate	0.25
	Hydrogen†	2.4
Carbon recovery, %		93
Oxidation-reduction index		1.18

* Initial and final ethanol concentration, 20.4 and 8.2 mM per 100 ml., respectively; initial and final propionate concentration, 14.6 and 4.6 mM per 100 ml., respectively.

† Determined in a small scale parallel experiment.

Ethanol-Butyrate Fermentation—Butyrate is not readily attacked by growing cultures of *Clostridium kluyveri* unless a little acetate is also present. For this reason acetate was added to the medium used for the ethanol-butyrate fermentation (Table V). The culture was allowed to incubate until growth ceased. The experiment was done on a small scale (25 ml.) because it was believed that the products would be qualitatively the same as those formed in an ethanol-acetate fermentation, and therefore large quantities were not required for the identification of the isomers. However, the presence of acetate, butyrate, and caproate in the fermented medium was established by separating the acids by azeotropic distillation and identifying the individual components by Duclaux distillation. Quantitative data on this fermentation are given in Table V. It is evident that most of the butyrate has been converted to caproate. The results will be discussed further below.

TABLE V

Ethanol-Butyrate Fermentation

		mM per 100 ml.
Unfermented medium	Ethanol	~10.00
	Acetate	1.00
	Butyrate	3.84
Fermented " *	Ethanol	3.06
	Acetate	1.01
	Butyrate	0.50
	Caproate	4.27

* H_2 was produced, but was not collected.

TABLE VI

Influence of Ethanol-Acetate Ratio on Yields of Butyrate and Caproate

		Experiment 1	Experiment 2	Experiment 3	Experiment 4
		mM per 100 ml.	*mM per 100 ml.*	*mM per 100 ml.*	*mM per 100 ml.*
Unfermented medium*	Ethanol	4.70	4.70	4.70	4.70
	Acetate	0.74	1.50	3.02	6.00
Fermented medium	Ethanol	2.95	2.08	0.64	0.64
	Acetate	0.91	0.62	0.84	2.82
	Butyrate	0.00	0.185	0.76	1.46
	Caproate	0.33	0.99	1.43	1.06
Carbon recovery, %		89	94	95	93

* Medium 1.

Influence of Ethanol-Acetate Ratio on Yield of Butyrate and Caproate—Preliminary experiments showed that the relative yields of butyrate and caproate are dependent upon the concentrations of the two substrates, ethanol and acetate. To examine this effect more carefully four media were made containing the same concentration of ethanol and four different concentrations of sodium acetate. The cultures were incubated until growth ceased and were than analyzed. The results are given in Table VI. It can be seen that when there is an excess of ethanol (Experiments 1 and 2), caproic acid is the main product and little or no butyric acid accumulates. As the excess of ethanol is reduced by increasing the acetate concentration, butyric acid becomes relatively and absolutely more abundant (Experiment 3); when acetate is present in excess, butyric acid accumulates in a larger amount than caproic acid and the absolute yield of caproic acid is decreased (Experiment 4).

DISCUSSION

The experiments described above show that *Clostridium kluyveri* is able to convert ethanol and acetate under anaerobic conditions almost quantitatively to *n*-butyrate, *n*-caproate, and hydrogen. Similarly, it converts ethanol and propionate to acetate, *n*-butyrate, *n*-valerate, *n*-caproate, *n*-heptanoate, and hydrogen. The experiments also provide evidence concerning the nature of some of the reactions involved in these fermentations.

Let us first consider the ethanol-acetate fermentation. If we disregard the production of hydrogen gas, the formation of butyrate and caproate from ethanol and acetate may be written formally as follows:

$$CH_3CH_2OH + CH_3COO^- = CH_3CH_2CH_2COO^- + H_2O \tag{1}$$

$$2CH_3CH_2OH + CH_3COO^- = CH_3(CH_2)_4COO^- + 2H_2O \tag{2}$$

Equation 1 represents the main reaction in a culture containing an excess of acetate (Table VI, Experiment 4). When, on the contrary, ethanol is present in excess and acetate is the limiting factor, little or no butyrate is formed and the fermentation proceeds according to Equation 2; this condition is realized approximately in Experiment 2 of Table VI. The fact that butyrate is the main product when ethanol is limiting and caproate is the main product when ethanol is in excess indicates that butyrate is an intermediate in the conversion of acetate to caproate. This indication is further supported by the observation that when butyrate is provided as a substrate it is almost completely converted to caproate (Table VI). All the evidence is consistent with the view that acetate is first converted to butyrate (Equation 1), which is then converted to caproate (Equation 3). However, there is as yet no proof that butyrate is an obligatory intermediate in the formation of caproate.

$$CH_3CH_2OH + CH_3(CH_2)_2COO^- = CH_3(CH_2)_4COO^- + H_2O \tag{3}$$

The mechanism of Equations 1 and 3 certainly is not a direct condensation, with a loss of water between the hydroxyl group of the alcohol and the methyl group of the fatty acid. Experiments with isotope-labeled acetate have shown that during the ethanol-acetate fermentation the ethanol is oxidized to acetic acid before a condensation occurs (10). This implies that this fermentation, like all others that have been studied, is an oxidation-reduction process. The reductants are ethanol and, possibly, acetaldehyde. The oxidant in Equation 1 has not been identified, but may be assumed to be a C_4 compound, such as acetoacetate, which is formed by the condensation of 2 molecules of acetic acid or an "energy-rich" derivative thereof and is capable of being reduced to butyric acid. A similar C_6 intermediate can be postulated as a precursor of caproic acid. In the oxidation of ethanol to acetate, 4 electrons are removed and these are just sufficient to reduce the hypothetical intermediate compounds to butyrate or caproate.

Equations 1, 2, and 3 give a simplified picture of the ethanol-acetate fermentation and do not describe accurately the observed quantitative relations among the substrates and products. The main complicating factor is the formation of hydrogen which modifies the simple oxidation-reduction relations that would otherwise exist. Hydrogen evolution provides a mechanism for the oxidation of an additional quantity of ethanol to acetic acid (Equation 4). As a result, the apparent utilization of acetic

$$CH_3CH_2OH + H_2O = CH_3COOH + 2H_2 \tag{4}$$

acid is always less than is to be expected from Equations 1 and 2. Another consequence of hydrogen production is that the total volatile acidity must increase during the fermentation. Both of these effects can be observed in the data of Tables II and VI.

The ethanol-propionate fermentation is fundamentally similar to the ethanol-acetate fermentation, but is complicated by the fact that both C_2 and C_3 compounds are involved. The main process is the conversion of ethanol and propionate to *n*-valerate (Equation 5), which undoubtedly involves an oxidation of ethanol to acetic acid, a condensation of a C_2 and a C_3 compound to form a C_5 intermediate, and a reduction of the intermediate to *n*-valeric acid.

$$CH_3CH_2OH + CH_3CH_2COO^- = CH_3(CH_2)_3COO^- + H_2O \tag{5}$$

The oxidation of ethanol to acetic acid is more obvious in the ethanol-propionate fermentation than in the ethanol-acetate fermentation because there is an accumulation rather than a net utilization of acetate. Also butyrate and caproate are formed in appreciable quantities. The butyrate is undoubtedly formed from acetate, just as it is in the ethanol-acetate fermentation. In the latter, only part of the acetate for butyrate synthesis was derived from alcohol, while in the ethanol-propionate fermentation all the acetate must have come from this source. The only other possible source of acetate is propionate, and this is excluded by the fact that no carbon dioxide was formed. The caproate also was probably derived ultimately from ethanol via acetate and butyrate, although the possibility that caproate can be formed from 2 molecules of propionate is not excluded.

Heptanoic acid is probably formed from valeric acid by a further condensation with a C_2 compound (Equation 6). It is evident from the rela-

$$CH_3CH_2OH + CH_3(CH_2)_3COO^- = CH_3(CH_2)_5COO^- + H_2O \quad (6)$$

tive amounts of valerate, caproate, and heptanoate (Table IV) that the conversion of valerate to heptanoate is much slower than the conversion of propionate to valerate or even the conversion of butyrate to caproate. Exact comparisons of the rates of utilization of propionate, butyrate, and valerate cannot be made from the available data, but there can be no doubt that the rates decrease rapidly with increasing chain length. Fatty acids containing more than 5 carbon atoms apparently are not utilized at a significant rate in catabolic reactions by the strain of *Clostridium kluyveri* which we have so far studied. This does not mean that the ability to synthesize longer chain fatty acids is altogether lacking. Although direct experimental data are not available, it is very probable that such compounds are formed as cell constituents by *Clostridium kluyveri*, just as they are by other bacteria.

Nutritional experiments have shown that *Clostridium kluyveri* grows very well in media containing ethanol and acetate or propionate as the only organic compounds present in appreciable quantities (4). It must be concluded that the reactions discussed in this paper (Equations 1 to 6) provide all the energy required for its vital activities. It is therefore of interest to estimate the amounts of energy available from these reactions. For this purpose Equation 1 may be taken as the type reaction. Free energy data are available for the reactants and products of this reaction (11, 12). To obtain an approximate value for the free energy change it is assumed that the ethanol and the fatty acid anions are present as 1 M solutions. The value for the free energy of formation of the butyrate ion was obtained from the value for liquid butyric acid by assuming a difference equal to that between liquid acetic acid and the acetate ion, for which data are available. The calculated ΔF_{25° for Equation 1 is -11.7 kilocalories. The values for Equations 3, 5, and 6 should be of the same order of magnitude, while that for Equation 2, which involves 2 moles of ethanol, must be about twice as great. An ethanol-acetate fermentation proceeding according to Equation 2 furnishes almost half as much energy as the alcoholic fermentation of glucose.

The oxidation of ethanol to acetate with the liberation of hydrogen gas (Equation 4) is calculated to have a ΔF_{25° of approximately zero at pH 7 in a bicarbonate buffer. From this result it might be expected that the evolution of hydrogen would stop before the pressure reached 1 atmosphere. However, it should be noted that the oxidation of ethanol probably involves two steps, one from ethanol to acetaldehyde, the other from acetaldehyde to acetate. If the hydrogen is assumed to be associated only with the second step (Equation 7), the ΔF_{25° becomes -4.9 kilocalories,

$$CH_3CHO + H_2O + HCO_3^- = CH_3COO^- + H_2 + H_2CO_3 \qquad (7)$$

which is large enough to account for the evolution of hydrogen gas.

SUMMARY

Clostridium kluyveri has been shown to convert ethanol and acetate more or less quantitatively to *n*-butyrate, *n*-caproate, and hydrogen and to convert ethanol and propionate to acetate, *n*-butyrate, *n*-valerate, *n*-caproate, *n*-heptanoate, and hydrogen. No carbon dioxide is formed. Evidence is presented to show that these fermentations are oxidation-reduction processes in which ethanol is oxidized to acetic acid and the higher fatty acids are formed by successive condensation and reduction reactions. Thermodynamic data show that these synthetic reactions are exergonic, and nutritional experiments have demonstrated that they are capable of supplying the energy needs of the bacteria.

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ENZYMATIC DIGESTION OF SAMPLES FOR MICROBIOLOGICAL ASSAY OF PANTOTHENIC ACID

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Since most natural materials contain a part of their pantothenic acid in a combined state which is not utilized by the test organism, a preliminary treatment of the sample is necessary before a microbiological assay will indicate the total vitamin content. Various enzymes have been employed (1–4) for the preparation of samples for assay; however, few have proved satisfactory, mainly because of their slow rate of action and their high content of pantothenic acid, which must be subtracted as a blank.

We have used mylase P[1] in a 2 to 3 hour digestion period (3) for the liberation of pantothenic acid from the combined state in samples for the past 7 years with good results. Recently Ives and Strong (5) showed mylase P to be superior to other enzymes and recommended its use; however, they felt that a 2 hour incubation period was inadequate for maximum liberation and that a 12 to 24 hour period was more desirable.

In view of these conflicting reports, we wish to present further data concerning the optimum conditions for the use of mylase P.

EXPERIMENTAL

A dried yeast extract was used as a substrate for the enzyme studies because it contained a high percentage of combined pantothenic acid. The activity of the lot of enzyme used in these experiments was determined (3). This was accomplished by finding the optimum ratio of enzyme to a 0.4 gm. sample of the yeast extract which would give maximum liberation of pantothenic acid in a 2 hour period at 50° in a total volume of 11 ml. at pH 4.5. This ratio was found to be 1 part of enzyme to 1 part of sample. The pantothenic acid content of this lot of mylase P was found to be 1.5 γ per gm. and the blank was determined by the ratio of enzyme to sample. Deviations from this standard procedure were made to determine the effect of pH, temperature, time, and concentration on the digestion process. All samples were filtered at pH 4.5 after digestion, as recommended by Ives and Strong (5), properly diluted, and assayed according to the method of Hoag *et al.* (6).

pH of Digestion—In order to determine the optimum pH for the digestion, an experiment was set up in which reaction mixtures were adjusted to various pH values. A stock solution containing 100 mg. of dried yeast extract per ml. was prepared in 1 M sodium acetate. 1 gm. aliquots were titrated to various pH values with HCl or NaOH and made to a volume of 20 ml. Aliquots containing 0.4 gm. were digested with 0.4 gm. of enzyme at 50° for 3 hours in a volume of 11 ml. After incubation all samples were readjusted to pH 4.5 before filtration. Maximum liberation was obtained at pH 4.5; however, it appears that the optimum pH range is quite broad.

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[1] Mylase P (special) for thiamine determination, obtained from the Wallerstein Laboratories, 180 Madison Avenue, New York.

pH	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Pantothenic acid per gm., γ	413	420	445	410	353	195	133	106

Temperature of Digestion—Since various investigators have used different temperatures for the enzymatic liberation of pantothenic acid, it was deemed desirable to investigate the effect of temperature. It has been recognized that an increase in temperature causes an increase in the activity of an enzyme system; however, this increase in temperature could cause destruction of the enzyme or the product liberated.

Table I

Effect of Varying Temperature of Mylase Digestion on Liberation of Pantothenic Acid

0.4 gm. digested with an equal part of mylase P in a volume of 11 ml. containing 2 ml. of 2 M acetate buffer, pH 4.5, for a period of 3 hours.

Sample	Pantothenic acid per gm.				
	37°	40°	45°	50°	55°
	γ	γ	γ	γ	γ
Dried yeast extract	418	425	459	495	434
Brewers' yeast	68	69	74	79	73
Whole liver (dried)	93	98	103	117	93

In this experiment two additional substrates were used; namely, brewers' yeast and dried whole liver. For each temperature a bottle was prepared containing 0.4 gm. of sample with an equal part of mylase P in a volume of 11 ml., containing 2 ml. of 2 M acetate buffer at pH 4.5. The mixtures were allowed to digest for a period of 3 hours at the various temperatures.

Table I shows typical results of carrying out the enzymatic digestion at temperatures ranging from 37–55°. The greatest liberation for the 3 hour period was obtained at 50°.

Incubation Period and Concentration of Digestion Mixture—Because other investigators (5) have obtained different results with the mylase digestion, experiments were set up to show the effect of the ratio of sample to enzyme, and the effect of the concentration of the digestion mixture on the time for complete liberation of the pantothenic acid. The experiment was planned to cover a complete range of dilutions with two ratios of sample to enzyme (1:1 and 10:1). The digestions were prepared from a solution of dried yeast extract containing 5 ml. of 2 M sodium acetate buffer per gm. of sample and adjusted to pH 4.5 with hydrochloric acid. The mylase P was added to the yeast extract solution just before the aliquots were placed in the digestion tubes. Since pipetting was carried out as rapidly as possible, little time elapsed before the incubation was started. The volume of each tube was adjusted with distilled water to correspond to the values indicated in Table II. Toluene was used as a preservative for long period digestions. The incubation periods were 2, 4, 6, and 20 hours. A separate tube was prepared for each time interval. At the end of each digestion period the tubes were removed and cooled immediately to 4° and stored at that temperature until used for assay the next day. Digestion was halted by refrigeration rather than by heat in order to minimize the destruction of pantothenic acid. The possibility of further digestion in the refrigerator was investigated and found to be negligible. Table II gives the results of the average of three separate runs of the same experiment.

TABLE II

Pantothenic Acid Liberated by Digestion with Two Concentrations of Mylase P at Different Volumes and Varying Lengths of Time

Two stock solutions containing 10 gm. of dried yeast extract and 50 ml. of 2 M acetate buffer were prepared and adjusted to pH 4.5 with HCl. 10 gm. and 1 gm. of mylase P were added to solutions (*a*) and (*b*) respectively and each made to a volume of 100 ml. Aliquots containing the desired weight of sample were immediately taken and made to the indicated volume and incubated at 50°. The original solutions were diluted 10-fold before the last two levels were prepared. Samples digested with (*a*) an equal weight of mylase P, (*b*) with one-tenth the weight of mylase P.

Digestion volume	Sample weight	Ratio of sample to volume	Pantothenic acid per gm.							
			2 hrs.		4 hrs.		6 hrs.		20 hrs.	
			(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
ml.	*gm.*		γ	γ	γ	γ	γ	γ	γ	γ
5	0.5	1:10	467	229	435	285	437	329	462	357
10	0.4	1:25	468	279	442	342	447	347	449	387
20	0.4	1:50	457	297	455	324	443	358	445	407
10	0.1	1:100	449	253	465	284	488	321	439	347
25	0.1	1:250	454	209	474	256	449	311	454	347
40	0.04	1:1000	470	188	454	196	476	220	484	279
40	0.02	1:2000	461	171	460	180	451	187	466	235

It will be noted that with adequate enzyme (column (*a*) of Table II) a 2 hour digestion period is sufficient; a longer digestion period gave no increase in pantothenic acid liberated and dilution of the digestion mixture did not decrease it. However, with less enzyme (column (*b*) of Table II) a longer digestion period was necessary to liberate the pantothenic acid. Furthermore, it was observed that as the digestion mixture was diluted a correspondingly increased time was required, as evidenced by the amount of pantothenic acid liberated at the respective dilutions and time intervals. With this ratio of sample to enzyme (10:1) the optimum volume for digestion appeared to be such that the ratio of sample to volume was between 1:25 and 1:50.

Recommended Procedure—Place 0.4 gm. of finely ground sample with 0.4 gm. of mylase P (the quantity depending upon the potency) in a 50 ml. tube and add 10 ml. of 2 per cent acetic acid and 1 ml. of 1 N NaOH, which buffers the reaction at pH 4.2 to 4.5. In the case of liquids or suspensions any organic solvent such as alcohol is removed. Aliquots containing about 0.4 gm. of material are diluted or concentrated to a volume of 10 ml., to which are added 0.2 ml. of glacial acetic acid, 1 ml. of N NaOH, and 0.4 gm. of mylase P. The mixture is allowed to digest 2 to 3 hours in a controlled water bath at 50°. At the end of the digestion period the samples are made up to 250 ml. with distilled water and filtered through No. 2 Whatman paper. (This filtration at pH 4.5 removes some interfering materials that may cause variation in the assay.) An aliquot of the clear filtrate is used to make a subsequent dilution containing about 0.02 to 0.04 γ of pantothenic acid per ml. In calculating the results it is necessary to consider the pantothenic acid contained in mylase P. This is determined by assaying mylase P and using this value as a blank. Most lots of mylase P have shown a very low blank and therefore could be ignored except when low potency material is being assayed.

DISCUSSION

The data here presented, supplementing a previous report (3), illustrate that the prime factor in the mylase P digestion is the use of an adequate quantity of enzyme. It is important that each lot of enzyme be tested for activity so that the correct amount may be employed. The essential difference in our method and that of Ives and Strong (5) is in the quantity of enzyme used for digestion. Their method called for mylase P at one-tenth the weight of the sample. Data presented in Table II compare the results of this level of enzyme (*b*) with a level equal to the weight of the sample (*a*). The data illustrate that the activity of the enzyme is of greater moment than the length of digestion period, because even in 20 hours with enzyme at a level of one-tenth the weight of the sample maximum results were not obtained.

Since mylase P reacts on other material besides combined pantothenic acid, enough enzyme should be supplied to allow full liberation of pantothenic acid in any material; thus it was deemed advisable to calculate the quantity of enzyme used on the basis of sample weight rather than on the content of pantothenic acid. Mylase P contains only a small quantity of pantothenic acid and hence blank corrections will be sufficiently low in most cases. In very low pantothenic acid-containing materials it may be desirable to have a lower blank which can be achieved by dialyzing the mylase P (3).

SUMMARY

A method is described for the enzymatic liberation of pantothenic acid by digestion with mylase P over periods of 2 to 3 hours. Studies were made on the factors affecting the mylase digestion, including pH, temperature, incubation period, volume, and ratio of sample to enzyme. The importance of determining the activity of each lot of enzyme before it is used for assay is demonstrated.

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STUDIES ON CHOLINE ACETYLASE

IV. EFFECT OF CITRIC ACID*

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Previous investigations have revealed that the formation of acetylcholine by the enzyme choline acetylase requires for maximum activity, in addition to the protein moiety, the following components: K^+, Mg^{++}, adenosine triphosphate (ATP), a coenzyme, cysteine, and eserine (1–5). The coenzyme, described simultaneously by Nachmansohn and Berman and Lipmann and Kaplan (5, 6), contains apparently pantothenic acid and seems to be the same for all acetylations (7). The cysteine is necessary for keeping the easily oxidizable —SH groups of the enzyme in a reduced state, especially if the enzyme has been prepared from acetone-dried powder. Eserine has to be added, since in most preparations some cholinesterase activity still remains, interfering with the acetylcholine formation.

Shortly after the discovery of choline acetylase by Nachmansohn and Machado in 1943, it was found that the enzyme extracted from fresh rat brain becomes rapidly inactive on dialysis (8). This observation suggested either the necessity of a coenzyme or of a precursor of acetate. On addition of either citric or L-glutamic acid in rather high concentrations (2×10^{-2} M), the enzyme could be partly reactivated (8, 3). This effect was later confirmed and its study was extended by other investigators (9, 10), but it has not yet been explained satisfactorily. Lipton and Barron proposed as a working hypothesis that citric acid may act as a donor of "active acetate," according to the equation citrate $\leftrightarrows$ oxalacetate + acetate (active). Acetate may be the degradation product of citrate as well as of other precursors, but there is no evidence that this is the way citric acid acts under the experimental conditions used and that the acetylation of choline requires the presence of "active acetate." In experiments in which isotopic acetate was used, most of the acetate of the acetylcholine formed, examined after crystallization as the Reinecke salt, contained isotope.[1] This result shows that acetate may be used directly and that there is no need to postulate an "active acetate" for the acetylation of choline. Moreover, a nearly (and often quite) complete reactivation of the enzyme prepared from acetone-

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[1] Bloch, K., Berman, M., and Nachmansohn, D., in preparation.

dried powder was obtained after dialysis if purified coenzyme was added. In that case, citric acid did not increase the rate of formation. The mode of action of citric acid and L-glutamic acid on acetylation of choline remains thus unexplained and requires further investigation.

It appeared interesting to test the effect of these compounds in a highly active enzyme system. Such a preparation may be obtained either by purification or by extracting the enzyme from a tissue with a high rate of acetylcholine metabolism. A high rate of formation may be assumed when the concentration of cholinesterase is high, since it is probable that there is a relation between these two rates. An unusually active tissue in this respect is the head ganglion of squid in which 1 gm. of fresh tissue may split about 3 gm. of acetylcholine per hour, or about 30 times as much as a rabbit brain.

In this paper a highly active choline acetylase preparation extracted from the acetone-dried powder of the head ganglion of squid will be described as well as its reaction with some compounds used previously in less active preparations.

Methods

Preparation of Enzyme—The acetone-dried powder of the head ganglion of squid was prepared in the same way as that of mammalian brain (3). One preparation may be described as an example. 17.5 gm. of ganglia obtained from about thirty squid were homogenized in 90 cc. with the usual ice-cooled phosphate-K^+ mixture and poured into 2.5 liters of acetone cooled to about −2°. The precipitate was left for 10 minutes in the acetone, then filtered with suction through a Büchner funnel. The solid was poured again into 2 liters of acetone. The precipitate was left this time for 30 minutes in acetone, then filtered, and dried in the usual way. The yield was about 1.5 gm. of powder.

The enzyme was extracted from the powder with the usual buffer solution. In the first experiments, the extract was centrifuged at 11,000 R.P.M. in the Serval angle head centrifuge. This high speed has proved useful for the extraction of acetone-dried powder of mammalian brain and, in the experiments described in this paper with mammalian brain enzyme, the high speed centrifugation was always applied. With the powder of squid ganglion, however, no difference was found whether the extraction occurred at 11,000 or at 5000 R.P.M. and therefore the lower speed was used in the later experiments.

Whereas in previous experiments, the total volume in the vessel varied from 4.5 to 5.0 cc., in the experiments described in this paper the volume used was usually between 3.2 and 3.5 cc. The following volumes of the different components were put into the vessel: 1.6 cc. of the enzyme, 0.1 cc.

each of eserine, acetate, and choline, 0.3 cc. of cysteine, and 0.6 cc. of coenzyme. 0.2 cc. of ATP was placed in the side bulb as usual. The coenzyme was prepared as powder from hog liver.[2] The 0.6 cc. of the coenzyme solution contained 2 mg. of substance, which was found repeatedly to be sufficient for maximum activity. The final concentrations of all compounds were essentially the same as described previously and the removal of the protein and the bioassay of the ester formed were also carried out in the usual way (5).

Results

The high rate of acetylcholine formation in extracts prepared from acetone-dried powder of the head ganglion of squid is shown in Table I.

TABLE I

Choline Acetylase from Head Ganglia of Squid

Extracts prepared from acetone-dried powder. Choline, acetate, cysteine, and eserine present in all vessels.

Experiment No.	Powder per vessel	Additions	Incubation	Acetylcholine formed		Enzyme activity*
				Per vessel	Per gm. per hr.	
	mg.		*min.*	γ	*mg.*	
1	10.0	None	10	0	0	
		ATP	10	21.0	12.6	
		" + coenzyme	10	56.0	33.6	150.0
2	14.5	None	15	0	0	
		ATP	15	50.0	13.8	
		" + coenzyme	15	108.0	29.2	139.0

* Activity of the enzyme per gm. of protein in mg. of acetylcholine formed per hour.

In extracts prepared from powder of acetone-dried rabbit brain in the same way and used in the same conditions except for the higher temperature of incubation (37°), 2.0 to 2.5 mg. of acetylcholine may be formed per gm. per hour. Since the extract from the head ganglion may form more than 30 mg., this rate is higher by about 12 to 15 times. If we take into consideration the difference of the Q_7, which for the energy-requiring synthesis is about 2 (3) and for the hydrolysis only about 1.5 (11), the rate of formation is indeed not far from the order of magnitude which could be expected on the basis of the rate of acetylcholine hydrolysis.

The degree of purity is also remarkably higher than in the extracts prepared from rabbit brain, even after high speed centrifugation. In two experiments, 1 gm. of protein could form 139 and 150 mg. of acetylcholine per hour respectively, compared with 10.4 mg. obtained as the highest value with the former preparation.

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[2] We are greatly obliged to Dr. L. A. Sweet and Dr. G. J. Mannering of Parke, Davis and Company, who kindly prepared the coenzyme for us.

The effect of ATP is particularly strong in these experiments in which the amounts of powder used are small, equivalent to 10 to 15 mg. per vessel. The concentration of acetylcholine formed after incubation for 10 to 15 minutes is 1 to 2×10^{-4} M, which is about one-third of the final value obtained after an incubation period of 60 minutes. The initial concentration of ATP is about 1×10^{-3} M. The small amounts of acetylcholine occasionally found in the absence of ATP in experiments in which larger amounts of powders are used have obviously to be ascribed to the preformed ATP or phosphocreatine phosphorylating adenosine diphosphate to ATP.

TABLE II

Reactivation of Dialyzed Choline Acetylase Prepared from Acetone-Dried Powder of Squid Head Ganglia

ATP, choline, acetate, and eserine always present in the usual concentrations. The time of dialysis was 2 hours.

		Acetylcholine formed per gm. per hr.
		mg.
Before dialysis	K, Mg, coenzyme, cysteine	22.4
Dialyzed	No additions	0
	K	1.4
	“ Mg	1.7
	“ “ coenzyme	2.1
	“ “ cysteine	7.2
	“ “ “ coenzyme	22.8
	“ “ “ glutamic acid	6.9
	“ “ “ “ “ coenzyme	22.7

The activity of this powder decreased slowly. Within about 2 weeks, the rate of acetylcholine formation fell to about 22 mg. per gm. per hour. Another powder capable of forming 38.8 mg. per gm. per hour immediately after preparation lost one-half of its activity within a few days but then remained nearly unchanged for several weeks.

Dialysis—On dialysis the enzyme rapidly lost all activity. If choline, acetate, eserine, and ATP were added, no reactivation was obtained (see Table II). But by adding K^+, Mg^{++}, coenzyme, and cysteine, a complete reactivation was obtained. L-Glutamic acid had no effect.

Effect of Acetic Acid—Whereas, in previous experiments, addition of choline proved always to be necessary, that of acetate had either a weak effect or none. This could be interpreted as being due to the presence of acetate in sufficient concentrations even in dialyzed preparations, since the amounts required are small and a prolonged dialysis was impossible, owing to the rapid deterioration of the enzyme.

In view of the high activity of the present preparation, the effect of addition of acetate on the reaction was tested. As Table III shows, this increased the rate considerably, suggesting that the acetate is used for the formation of acetylcholine. With concentrations ranging from 7.5×10^{-3} to 6×10^{-2} M, the rate of formation was raised by 60 to 65 per cent (Experiment 2). Lower concentrations were not tested for lack of material. Addition of acetylglycine had no effect in 5×10^{-2} M concentration.

TABLE III

Effect of Acetic Acid and Acetylglycine on Choline Acetylase Prepared from Acetone-Dried Squid Ganglia

In addition to the enzyme, coenzyme, K, Mg, choline, eserine, and ATP are present in all vessels.

Experiment No.	Compound	Concentration	Acetylcholine formed per gm. per hr.	Increase
		M × 10^{-2}	*mg.*	*per cent*
1			3.34	
	Acetate	3	8.10	142.5
2			6.06	
	Acetate	6	10.00	65.0
	“	3	9.56	
	“	1.5	9.80	
	“	0.75	9.30	
3			3.34	
	Acetylglycine	5	3.76	
	Acetate	3	7.35	119.0

Citric Acid—Surprisingly, citric acid had, in this preparation, an inhibitory effect (Table IV), which was equally strong on dialyzed and undialyzed preparations. If to the dialyzed solution, all the usual additions were made without citrate, a complete reactivation was obtained. The degree of inhibition varied slightly in the different experiments. In Experiment 1, with undialyzed solution, 30.5 per cent inhibition was still obtained with 5×10^{-3} M concentration. The inhibition could not be overcome by raising the ATP or the coenzyme concentration (Table V).

Inhibitors of Choline Acetylase—Two kinds of compounds were found in previous observations to inhibit choline acetylase: α-keto acids and some naphthoquinones. The α-ketoglutaric acid was found to be the strongest inhibitor among the α-keto acids, effective in 10^{-4} M concentration, whereas among the naphthoquinones, 2-methyl-1,4-naphthoquinone-8-sulfonic acid (K salt), the strongest observed so far (5), had an inhibitory effect in concentrations as low as 3×10^{-5} M.

Table IV

Inhibition of Choline Acetylase by Citric Acid

Enzyme prepared from an acetone-dried powder of squid ganglia. Additions as usual (see Table III).

Experiment No.	Undialyzed				Dialyzed			
	Concentration	Acetylcholine formed per gm. per hr.		Inhibition	Concentration	Acetylcholine formed per gm. per hr.		Inhibition
		Control	Experimental			Control	Experimental	
	$M \times 10^{-2}$	mg.	mg.	per cent	$M \times 10^{-2}$	mg.	mg.	per cent
1	2.0	12.8	5.80	54.6	3.0	22.3	6.1	72.7
	1.0	12.8	7.45	41.8	2.0	22.3	7.9	64.6
	0.5	12.8	8.90	30.5	1.0	22.3	11.2	50.3
	0.25	12.8	11.0	14.1	1.5	22.8	13.7	39.9
2	2.0	17.1	10.1	41.0	2.0	8.8	5.2	41.0
3	2.0	8.8	5.8	29.3	1.0	8.8	6.3	28.4

Table V

Citric Acid Effect on Choline Acetylase in Presence of Increased Concentrations of ATP and Coenzyme

All other conditions as in Table IV.

Experiment No.	Concentration of citric acid	Compound varied	Concentration	Acetylcholine formed per gm. per hr.	Inhibition
	$M \times 10^{-3}$			mg.	per cent
1	0	ATP	2.5×10^{-3} M	12.3	
	5	"	2.5×10^{-3} "	7.5	39.0
	5	"	5.0×10^{-3} "	7.5	39.0
2	0	Coenzyme	2 mg. per vessel	14.5	
	5	"	2 " " "	12.1	16.5
	0	"	4 " " "	12.9	
	5	"	4 " " "	9.8	24.0
3	0	"	2 " " "	12.8	
	5	"	2 " " "	8.9	30.5
	5	"	4 " " "	8.5	33.5

In contrast to the effect on the preparation from mammalian brain, α-ketoglutaric acid had no measurable effect on the present preparation, even in a concentration of 5×10^{-3} M. The naphthoquinone compound had a strong inhibitory effect which proved to be of the same strength as on the extract prepared from the powder of acetone-dried rabbit brains (Fig. 1).

Ion Effects—In previous experiments (3, 5), the effect of potassium, magnesium, and manganese on choline acetylase activity was tested. It was found with potassium that maximum activity is obtained with a concentration close to 10^{-1} M. This high concentration is of the same order of magnitude as that found in nerve and muscle cells. Addition of magnesium in about 5×10^{-4} to 1×10^{-3} M concentration and manganese in about 10^{-4} M concentration still further increased the rate of formation, although only slightly (5). The necessity of the presence of one of these two ions is not surprising in view of their well known rôle in phosphorylation (see also "Discussion").

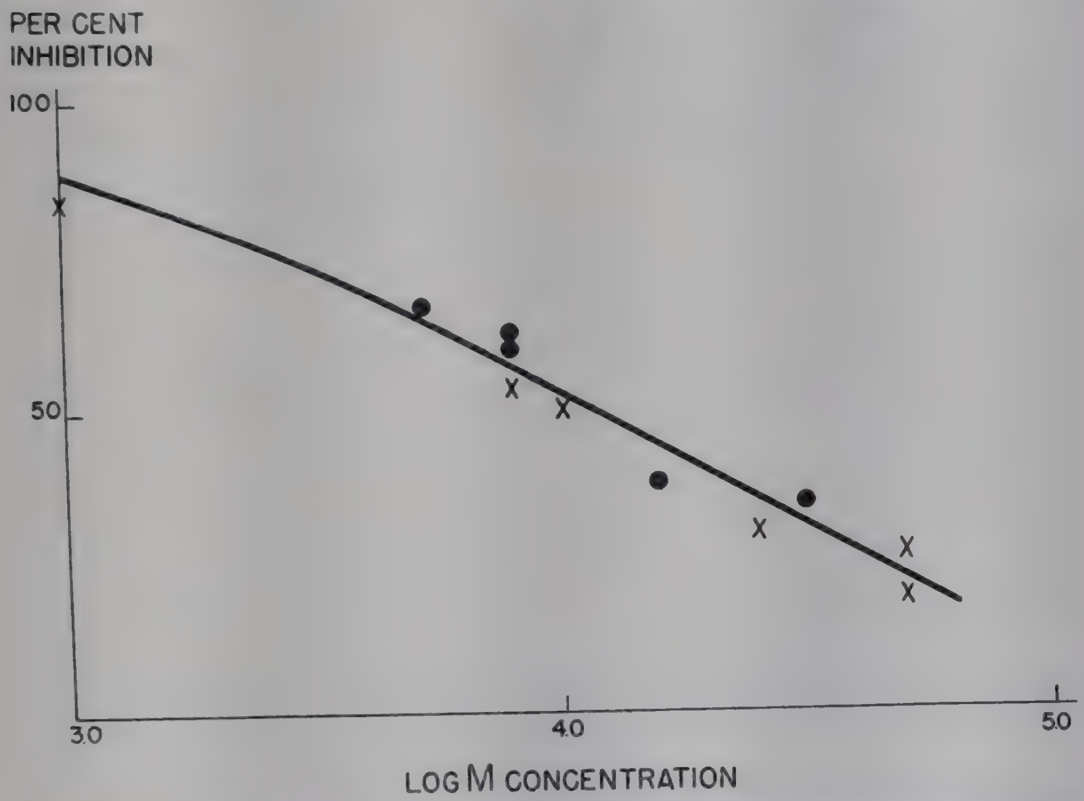

FIG. 1. Inhibitory effect of 2-methyl-1,4-naphthoquinone-8-sulfonic acid (K salt) on choline acetylase. Comparison of the degree of inhibition produced on an enzyme prepared from rabbit brain and on that prepared from the head ganglion of squid. ●, rabbit enzyme; ×, squid enzyme.

It appeared of interest to test the specificity of the potassium effect. It was found in experiments on enzyme preparations obtained from the acetone-dried powder of rabbit brain that potassium could be replaced by rubidium. Both ions have the same effect when used in the same concentration. In contrast, sodium and lithium have no effect. Two experiments with K^+, Rb^+, and Li^+, carried out on the same extract, are reproduced in Fig. 2.

Effect of Diisopropyl Fluorophosphate (DFP)—A great variety of facts have revealed that the high toxicity of DFP can be referred exclusively to its action on cholinesterase (11–15). The compound does not interfere with the choline acetylase activity *in vitro*. In the case of the electric tissue, it was even effectively used for increasing the yield of acetylcholine formed (5).

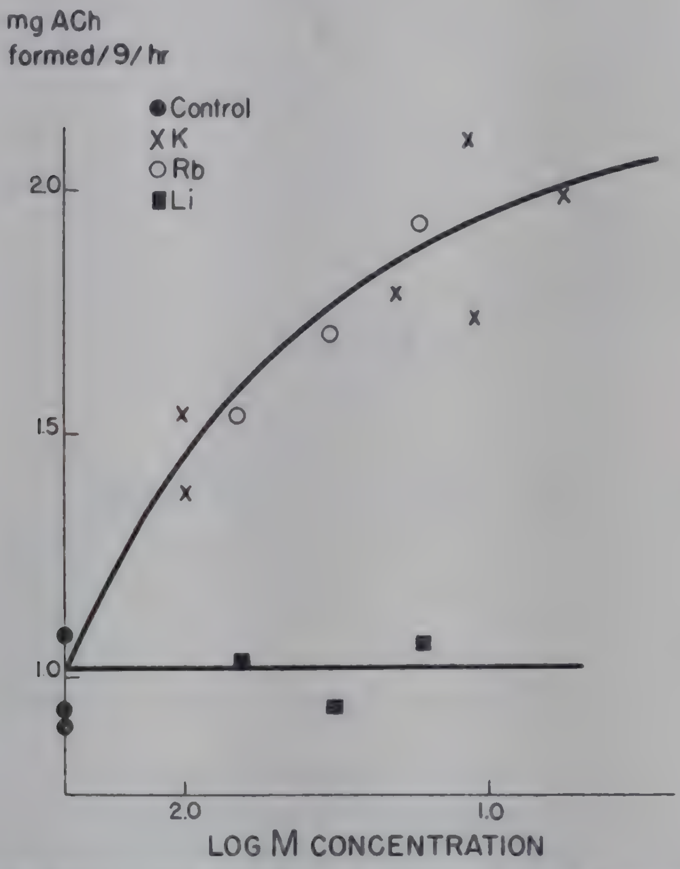

FIG. 2. Effect of potassium, rubidium, and lithium on choline acetylase prepared from acetone-dried powder of rabbit brain.

The choline acetylase activity in the brain of rabbits killed by DFP injection has now been tested. In these animals, no cholinesterase activity can be detected in brain tissue. The complete inactivation of this enzyme always coincides with death. It appeared desirable to know whether, under these conditions, when the toxic effect is so strong that death occurs the enzyme system responsible for the formation of acetylcholine remains unaffected.

Four powders were prepared from rabbit brains in the usual way after the rabbits were killed by injection of DFP, with 1 mg. of the compound per kilo of body weight. The acetylcholine formed per gm. per hour by these four powders was found to be as follows: 1.67, 1.69, 2.44, and 1.97 mg. These values are of the same order of magnitude as those obtained with powders from normal rabbit brains.

DISCUSSION

It is of interest to compare the reactions of the choline acetylase obtained from mammalian brain with those of the enzyme from squid ganglia, in which the rate of synthesis per gm. of protein is about 14 to 15 times as high as in the similarly prepared mammalian brain extracts. In an enzyme solution with a considerably higher degree of purity, other reactions will interfere to a much smaller extent than in less active extracts and the probability becomes greater that the reactions observed are intrinsic properties of the enzyme studied.

Among the differences between the new and the previous preparations, the effects of acetic and citric acids appear particularly noteworthy. Whereas addition of choline proved to be necessary in all preparations, acetic acid had only a negligible effect in the extracts used previously, even when they were dialyzed. Since the absolute amounts of acetic acid required are small, it appears possible that these small amounts are present in the extracts, even after dialysis carried out for a short period only. Moreover, as pointed out by Ochoa (personal communication), the ATP preparation usually contains small amounts of acetate owing to the use of barium acetate for the precipitation of the nucleotide and may therefore supply the acetic acid required. The strong effect of acetic acid added to the highly active enzyme preparation obtained from squid ganglia shows that the acetate is used by the enzyme for the acetylation directly and that, in this case, no "active acetate" is required.

It has, of course, to be kept in mind that the enzyme is extracted from a different source, *viz.* an invertebrate nerve tissue. It would be desirable to study this reaction in a choline acetylase preparation obtained from mammalian brain after purification. Such experiments are in progress. But the present observations are suggestive in connection with the previously mentioned observations; *i.e.*, the findings with the labeled acetate and the absence of the citric acid effect when a high rate of acetylcholine formation is obtained in the presence of an active coenzyme.

The inhibitory effect of the citric acid appears at first surprising, in view of the previous observations. It is possible that the citric acid removes the Mg ions necessary for the transphosphorylations. Mg is probably always present in small concentrations, even after a short dialysis. The weakness of the effect of added Mg is probably ascribable to the presence in the original extracts of Mg in amounts sufficient for a rate close to the maximum. The inhibitory effect of the citric acid is obtained with concentrations which are probably sufficiently high for the removal of the Mg ions added as well as of those originally present. The question may be raised why no inhibition is found when the citric acid is added in the same high concentration to the less active extracts. There, however, it may have reacted with calcium and other components not present here in sufficiently high concentration. Unfortunately, the interpretation could not be tested experimentally since no more material was available.

The similarity of the inhibitory effect of the 2-methyl-1,4-naphthoquinone-8-sulfonic acid (K salt) on the two enzyme preparations suggests a specific action on the enzyme protein. If it were an unspecific reaction, for example, with —SH groups, which in view of the high cysteine concentration appears unlikely, it would be difficult to explain why the degree of inhibition is the same in both cases. The observation increases still further the interest in the affinity of the naphthoquinones to choline acetylase. The action of these compounds, tested with a great variety of them, will be described in a separate paper.

SUMMARY

1. A highly active choline acetylase preparation has been obtained from acetone-dried powder of the head ganglia of squid. In these extracts up to 150 mg. of acetylcholine may be formed per gm. of protein per hour. The degree of purity referred to protein is about 14 to 15 times as high as in the best preparations obtained previously.

2. On dialysis, the enzyme activity falls rapidly to zero. On addition of choline, acetate, K, Mg, coenzyme, ATP, eserine, and cysteine, the total initial activity may be restored, suggesting that under these conditions the enzyme system is complete.

3. Whereas, in previous extracts, addition of acetate had either a weak effect on the synthesis or none, in this case the increase of the rate of formation was marked, showing that the acetate has been used directly for the acetylation.

4. In contrast to the effect observed in previous preparations, citric acid had an inhibitory effect, whereas α-ketoglutaric acid had no effect. The citric acid possibly removes the Mg ions necessary for transphosphorylations.

5. 2-Methyl-1,4-naphthoquinone-8-sulfonic acid (K salt) has the same effect on the highly active preparation described here as on the previous preparations extracted from mammalian brain. The similarity of these two effects suggests a specific reaction with the enzyme.

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THE RELATION BETWEEN CHEMICAL STRUCTURE AND FUNGICIDAL ACTION IN A SERIES OF SUBSTITUTED AND UNSUBSTITUTED PYRIDINIUM HALIDES*

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Although many publications have dealt with the bactericidal efficiency of different types of quaternary compounds, the literature on the use of these compounds as fungicides is still very limited. Horsfall (1) credits Howard and Keil (2) with the first publication on the quaternary compounds as fungicides.

Howard and coworkers (2–4) reported briefly on the fungicidal efficiency of the alkyldimethylbenzylammonium halides (alkyl = C_{10}–C_{18}), $[R(CH_3)_2C_6H_5CH_2N]^+X^-$, some monobasic derivatives of pyridine, picoline, lutidine, collidine, quinaldine, quinoline, and, finally, some quaternary derivatives of nicotine. A high degree of control against dollar spot (*Sclerotinia homeocarpa*) was obtained by Keil (5) by the use of dimethyldidodecylammonium chloride, while Daines and Hopperstead (6) found dodecylisoquinolinium bromide to be effective in providing protection against apple scab (*Venturia inaequalis*).

The purpose of the present study was to determine the relation between chain length and toxicity to fungi in the series hexyl-, octyl-, decyl-, dodecyl-, tetradecyl-, cetyl-, and octadecylpyridinium chlorides, and also the effect of alkyl substitutions in the 2 and 4 positions of the pyridine nucleus on the fungicidal efficiency of the resultant N-dodecyl-2- and N-dodecyl-4-alkylpyridinium bromides. Several of the compounds reported in this paper have not been previously described in the literature, and for this reason their biological properties should be of considerable interest.

EXPERIMENTAL

The alkyl chlorides were prepared from alcohols by the thionyl chloride method (7); prior to fractionation each was distilled in an ordinary Claisen flask, then washed three times with an equal volume of concentrated sulfuric acid, with water until neutral, and finally dried over anhydrous calcium chloride. Dodecyl bromide was made with concentrated hydrobromic and sulfuric acids (8); purification was accomplished in the same way as for the chlorides. The purified halides were distilled through a packed column having about fifteen plates with a total reflux, variable take off head, as described by Whitmore and Lux (9).

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The quaternary compounds were prepared by allowing the alkyl halides to react with pyridine or the substituted pyridines in all-glass pressure bottles at 135–140° in a thermostatically controlled oven for 36 to 48 hours. Purification was accomplished by repeated extraction with petroleum ether (b.p. 80°) or a mixture of it with ethyl ether (15 to 25 per cent) in which the quaternary compounds were insoluble. The salts were dried 5 to 6 hours in a vacuum oven at 80° at 35 to 40 mm. pressure. This is a modification of the procedure of Delépine (10). The analytical results (presented with the experimental data in Tables I to III) indicate that the compounds were in a high state of purity.

The toxicities of the pyridinium halides were determined by the slide germination method (11), with the conidia of *Sclerotinia fructicola*, *Venturia inaequalis*, and *Alternaria oleracea* and the swarm spores of *Phytophthora infestans*. Dosage-response curves were prepared for all compounds, and the LD_{50} values determined from these curves.

DISCUSSION

The first group of pyridinium compounds investigated in the present study consisted of derivatives of unsubstituted pyridine in which the length of the N-alkyl chain was varied from 6 to 18 carbon atoms (Table I). With one exception, all of the compounds in this series were chlorides.

From Table I it is apparent that there is a definite relation between the length of the N-alkyl group attached to the pyridine ring and the toxicity of the compound to the fungi. There were no significant differences between the slopes of the dosage-response curves for this series of compounds, and so the LD_{50} values may be considered to be a reliable indication of the relative toxicities within the group.

Of the individual compounds in this series, the N-hexyl derivative exhibited the least toxicity to the fungi. As the molecular weight of the N-alkyl group increased, there was a corresponding increase in toxicity up to and including the C_{14} derivative. Beyond this point, *i.e.* with alkyl groups containing 16 and 18 carbon atoms, toxicity to the fungi decreased slightly.

The marked increase in the toxicity of the lower members of the series is worthy of note, particularly in the three compounds with 6, 8, and 10 carbon atoms in the N-alkyl group. Increase in the molecular weight of the substituent group beyond the C_{10} compound resulted in less marked changes in toxicity. In order for a pyridinium chloride to possess superior toxicity to the fungi tested, it appears necessary that the N-alkyl group should contain at least 10 carbon atoms.

When the toxicity of dodecylpyridinium bromide is compared with that of the corresponding chloride, no significant differences are apparent.

TABLE I

Analytical and Fungicidal Data on Series of N-Alkylpyridinium Compounds

Compound	Halogen*		LD_{50}†			
	Calculated	Determined	*S. fructicola*	*V. inaequalis*	*P. infestans*	*A. oleracea*
	per cent	*per cent*				
Dodecylpyridinium bromide	24.39	24.41	0.29	0.076	0.28	
Hexylpyridinium chloride	17.82	17.81	100+	14.0	26.0	98.0
Octylpyridinium "	15.63	15.37	9.4	1.0	5.75	2.95
Decylpyridinium "	13.90	13.84	0.72	0.19	0.59	0.30
Dodecylpyridinium chloride	12.50	12.38	0.29	0.12	0.34	0.26
Tetradecylpyridinium chloride	11.40	11.13	0.17	0.06	0.10	0.22
Cetylpyridinium chloride	10.48	10.35	0.22	0.11	0.17	0.38
Octadecylpyridinium chloride	9.68	9.57	0.33	0.32	0.45	0.38

* Ionizable halogen by the Mohr method.

† Concentration of fungicide in micrograms per sq. cm. required to kill 50 per cent of conidia of *Sclerotinia fructicola*, *Venturia inaequalis* and *Alternaria oleracea* and the swarm spores of *Phytophthora infestans*.

TABLE II

Analytical and Fungicidal Data on Series of N-Dodecyl-2-alkylpyridinium Bromides

Compound	Halogen*		LD_{50}†		
	Calculated	Determined	*S. fructicola*	*V. inaequalis*	*P. infestans*
	per cent	*per cent*			
Dodecylpyridinium bromide	24.39	24.41	0.29	0.076	0.28
Dodecyl-2-ethylpyridinium bromide	22.43	22.10	0.27	0.037	0.28
Dodecyl-2-hexylpyridinium "	19.45	19.62	0.44	0.036	0.31
Dodecyl-2-heptylpyridinium bromide	18.77	18.85	0.16	0.032	0.29
Dodecyl-2(3-octyl)pyridinium bromide	18.18	22.10‡	0.22	0.036	0.18
Dodecyl-2(2-methyloctyl)pyridinium bromide	17.61	17.80	0.19	0.07	0.05

* Ionizable halogen by the Mohr method.

† Concentration of fungicide in micrograms per sq. cm. required to kill 50 per cent of the conidia of *Sclerotinia fructicola* and *Venturia inaequalis* and the swarm spores of *Phytophthora infestans*.

‡ High halogen indicates dehydrohalogenation occurred during the reaction with the formation of pyridine hydrobromide.

Of the four fungi used as test organisms in this experiment, *Venturia inaequalis* was least resistant to the compounds tested.

In order to determine the effect on toxicity of alkyl groups attached to carbon atoms in the pyridine ring, two additional series of compounds were prepared. In the first group (Table II) the parent compound may be considered to be dodecylpyridinium bromide. The other compounds in this group contained alkyl groups attached to the pyridine ring in the 2 position. The attached alkyl groups varied from C_2 to C_9, and included branched as well as straight chain alkyl groups.

It is apparent from the data in Table II that the introduction of an alkyl group in the 2 position in the pyridine ring caused no marked change in the toxicity of the dodecylpyridinium bromide. The effect of increased molecular weight in the 2-alkyl substituent groups was apparent in the LD_{50} values for one of the three test organisms, *Phytophthora infestans*. Increases in molecular weight increased the toxicity toward this fungus, and it may be that the point of maximum toxicity was not reached with the C_9 derivative. With the other test organisms, however, there appeared to be little effect on toxicity which could be attributed to the 2-alkyl substituent.

TABLE III

Analytical and Fungicidal Data on Series of N-Dodecyl-4-alkylpyridinium Bromides

Compound	Halogen*		LD_{50}†		
	Calculated	Determined	*S. fructicola*	*V. inaequalis*	*P. infestans*
	per cent	*per cent*			
Dodecylpyridinium bromide	24.39	24.41	0.29	0.076	0.28
Dodecyl-4-butylpyridinium bromide	20.80	20.15	0.64	0.084	0.20
Dodecyl-4-amylpyridinium "	20.08	19.60	0.34	0.084	0.33
Dodecyl-4(3-pentyl)pyridinium bromide	20.08	19.35	0.32	0.031	0.25
Dodecyl-4(5-nonyl)pyridinium bromide	17.61	17.50	0.21	0.07	0.05

* Ionizable halogen by the Mohr method.

† Concentration of fungicide in micrograms per sq. cm. required to kill 50 per cent of the conidia of *Sclerotinia fructicola* and *Venturia inaequalis* and the swarm spores of *Phytophthora infestans*.

The final series of compounds studied may be considered to be derived from dodecylpyridinium bromide with alkyl groups attached in the 4 position. Although this series includes only compounds with 4-alkyl groups ranging from C_4 to C_9, it appears that increases in molecular weight of the 4-alkyl groups resulted in increased toxicity to the fungi. As in the case of the 2-alkylpyridinium bromides, it is possible that further increases in molecular weight of the 4-alkyl group might have resulted in further increases in toxicity.

Comparison of the toxicity of dodecyl-4-amylpyridinium bromide (straight chain) with dodecyl-4(3-pentyl)pyridinium bromide (branched chain) indicates that there were no significant differences between the two (Table III). It should be noted further that the largest molecules in the two latter groups, both with C_9 substitution, had identical toxicities to the fungi, even though the degree of branching and the location of the substituent groups on the pyridine ring were different. This suggests that neither the degree of branching nor the position of the substituent (2- *versus* 4-) on the ring has as important an effect on toxicity as the molecular weight.

Of the organisms tested, *Venturia inaequalis* was the most susceptible to the action of the pyridinium halides. There were no significant differences in the slopes of the dosage-response curves.

Further quaternary derivatives of substituted pyridines are being prepared and tested against fungi. This work will be reported in subsequent papers.

SUMMARY

Three groups of pyridinium halides, comprising seventeen compounds, were prepared and tested against four species of fungi. In the first group, N-alkyl derivatives of unsubstituted pyridine, there was a significant relation between the length of the N-alkyl chain and toxicity, the maximum effect being observed in compounds having a C_{14} group. In the other two groups, 2- and 4-alkylpyridinium bromides, there were less marked variations in toxicity, although in general compounds having substituent groups of higher molecular weight showed the highest toxicity. There were no significant differences attributable to position of attachment or degree of branching of the substituent group attached to the carbon atoms of the pyridine ring.

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THE COLORIMETRIC DETERMINATION OF BLOOD CHLORIDE BY THE IODOMETRIC METHOD

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The iodometric method of Sendroy (1) for the determination of chloride as modified by Van Slyke and Hiller (2) leaves little to be desired as a volumetric procedure. A colorimetric method of comparable accuracy and simplicity may have some advantages, however, particularly when a number of determinations are to be done at the same time or the amount of material is small. Colorimetric methods based on the iodometric procedure have been described by Sendroy (3) and Hoffman (4), but they are not comparable to the volumetric method in simplicity and accuracy. The method presented in this communication is simple, accurate, and rapid. It utilizes an iodometric ion exchange identical with that of Van Slyke and Hiller; so that the same reagents are used and either the volumetric or colorimetric procedure, or both, may be performed on the same sample.

Reagents

With the exception of the 100 mM standard and the dilute thiosulfate reagent, the remaining reagents are the same as those of Van Slyke and Hiller (2). Their original paper should be consulted for a more complete discussion.

The *phosphoric-tungstic acid*, *0.9212 N thiosulfate*, *0.02303 N thiosulfate*, *silver iodate*, and *sodium iodide* are as specified by Van Slyke and Hiller (2).

100 mM chloride standard. Fill a 250 ml. volumetric flask to the mark with phosphoric-tungstic acid. To this add 10 ml. of 0.1 M NaCl.

Dilute thiosulfate reagent. Accurately measure 7.5 ml. of 0.02303 N thiosulfate into a 200 ml. volumetric flask and make up to volume with distilled water. This solution should be made fresh each day.

Procedure

(1) In a 10 ml. centrifuge tube place 5 ml. of phosphoric-tungstic acid. With an accurate wash-out pipette add 0.2 ml. of serum or plasma. (2) In a second centrifuge tube place 5 ml. of 100 mM chloride standard. (3) To each tube add approximately 60 mg. of silver iodate with a small glass spoon. Stopper with a rubber stopper and shake vigor-

ously for 40 seconds. Centrifuge for 1 minute at 2500 R.P.M. (4) To each of two colorimeter tubes (Evelyn colorimeter) add approximately 200 mg. of sodium iodide and 5 ml. of water. Transfer 0.5 ml. of the clear supernatant from each centrifuge tube to its respective colorimeter tube. Add 10 ml. of dilute thiosulfate reagent to each. Mix and read against a water blank at 420 mμ.

Calculation

$$\frac{25\ L_u}{L_s} + 75 = \text{mM Cl per liter}$$

where $L_u = (2 - \log G)$ for the serum and $L_s = (2 - \log G)$ for the standard.

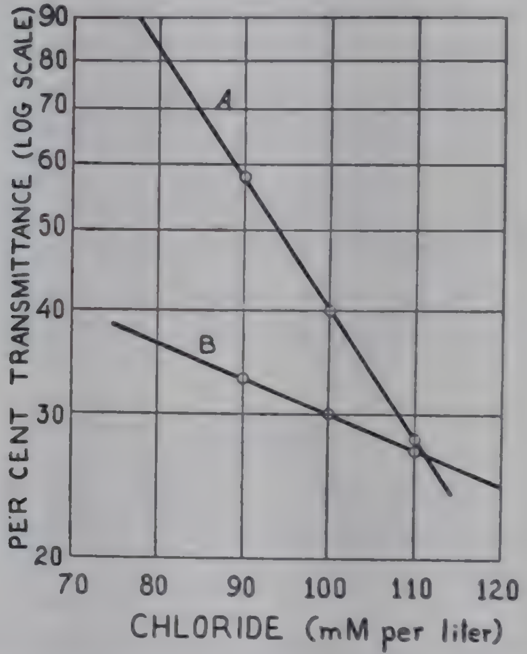

FIG. 1. Relation between chloride concentration and per cent transmittance Curve A, 75 mM of chloride removed; Curve B, 1:100 dilution. Evelyn colorimeter, Filter 420.

EXPERIMENTAL

Spectrophotometric studies indicate that the iodine color has a maximum absorption in the region of 400 mμ. The spectral transmission curve then slopes off to a minimum at 650 mμ. Proportionality curves were run at 10 mμ intervals from 400 to 650 mμ. There is practically no deviation from a straight line up to 430 mμ.

The effect of fading up to a period of 5 hours is negligible. The intensity of the color varies slightly with temperature, a fact noticed by Hoffman (4) and confirmed in this laboratory. For this reason a standard is run for each set of determinations instead of using a reference curve.

Sendroy and Hoffman have adapted the iodometric procedure to the colorimeter, bringing the final color into the range of the instrument by extensive dilution. This results in considerable loss of sensitivity as may be seen in Curve B, Fig. 1, where one scale division on the Evelyn colorimeter is equivalent to 3.3 mM of chloride. By removing the color produced by exactly 75 mM of chloride with thiosulfate, it is possible to determine the remaining 25 to 35 mM with a degree of sensitivity comparable to the best volumetric methods. Curve A in Fig. 1 shows the range obtained by this procedure: here one scale division on the colorimeter represents from 0.5 to 0.8 mM of chloride.

TABLE I

Comparison of Colorimetric Method with Volumetric Method of Van Slyke and Hiller

Colorimetric method	Volumetric method	Difference	Colorimetric method	Volumetric method	Difference
mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
84.9	81	+3.9	105.4	108	−2.6
91.0	94	−3.0	105.6	108	−2.4
91.3	90	+1.3	105.6	106	−0.4
96.0	94	+2.0	105.8	107	−1.2
97.2	94	+3.2	105.8	107	−1.2
99.6	97	+2.6	106.2	110	−3.8
101.0	98	+3.0	106.4	106	+0.4
101.0	101	0.0	106.8	107	−0.2
101.6	104	−2.4	106.8	106	+0.8
101.8	101	+0.8	106.9	107	−0.1
102.2	101	+1.2	107.1	106	+1.1
102.3	100	+2.3	107.1	106	+1.1
102.4	102	+0.4	107.1	104	+3.1
102.9	104	−1.1	107.6	108	−0.4
102.9	102	+0.9	107.8	109	+1.1
103.9	105	−1.1	108.0	108	0.0
104.0	102	+2.0	108.3	112	−3.7
104.0	104	0.0	108.3	112	−3.7
104.0	108	−4.0	108.8	109	−0.2
104.0	106	−2.0	108.8	110	−1.2
104.2	104	+0.2	109.1	110	−0.9
104.6	105	−0.4	109.8	110	−0.2
104.9	107	−2.1	110.1	109	+1.1
105.0	109	−4.0	110.8	112	−1.2
105.2	103	+2.2	113.0	114	−1.0
Average			104.20	104.54	±1.58

Table I gives a comparison of the results obtained by the colorimetric method described and the volumetric method of Van Slyke and Hiller with 1 ml. of serum. Normal and pathological sera are included, as well as sera to which sodium chloride solution or water has been added to increase or decrease the chloride content.

Both methods are subject to one inherent source of error common to procedures involving removal of protein by precipitation; namely, the volume error introduced by the protein precipitate. To determine the extent of this error, the protein precipitates from 1 ml. of four normal sera were dried to constant weight and their volumes determined by water displacement. The average weight of the precipitates was 0.1361 gm., and the average volume occupied was 0.0757 ml. This volume introduces an error of only about 0.3 per cent, which may be disregarded.

DISCUSSION

The essential feature of the colorimetric method described is a greatly increased sensitivity obtained by removing with thiosulfate the iodine produced by a standard amount of chloride before the final reading. The removal of iodine equivalent to 75 mM of chloride per liter of serum is suitable for most human sera, since values below this figure are seldom encountered. Under special circumstances the conditions may be varied by adding more or less 0.02303 N thiosulfate to the dilute thiosulfate reagent, 1 ml. of 0.02303 N thiosulfate being equivalent to 10 mM of chloride.

Any photoelectric colorimeter with provision for reading at a wavelength from 400 to 430 mμ can be used. The Evelyn macro colorimeter employs a 2 cm. cell; in instruments with a 1 cm. cell approximately twice the color intensity may be read. Adjustment for a 1 cm. cell thickness may be obtained by adding 2.5 ml. of water instead of 5 ml. and by using 5 ml. of a thiosulfate reagent made by diluting 7.5 ml. of 0.02303 N thiosulfate to 100 ml.

SUMMARY

A simple accurate colorimetric method for the determination of blood chloride is presented.

The method compares favorably with the volumetric method of Van Slyke and Hiller.

The author wishes to express his appreciation to Dr. Gladys J. Fashena for her helpful advice and suggestions.

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PLASMA ACCELERATOR GLOBULIN: PARTIAL PURIFICATION, QUANTITATIVE DETERMINATION, AND PROPERTIES*

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It has been shown that plasma contains a factor which accelerates the activation of prothrombin by thromboplastin and calcium ions (1–5). We refer to this factor as Ac-globulin (5).

In this paper we describe a method for the quantitative determination of Ac-globulin which is essentially an adaptation of the two-stage method for prothrombin analysis. Preparative methods are outlined which have yielded products which appear to be about 50 per cent pure by electrophoresis. Ac-Globulin is shown to be similar to prothrombin in many of its properties. Purified preparations of Ac-globulin have been found to be less stable towards heat than purified prothrombin. In oxalated bovine plasma, stored at 5°, Ac-globulin is quite stable.

Determination of Ac-Globulin Activity

Theory—When purified prothrombin, of the type described by Seegers, Loomis, and Vandenbelt (6), reacts with thromboplastin and calcium ions, according to the specifications of the two-stage technique (7–9), thrombin formation is very slow. It is now known that the slow activation rate is the result of the removal of Ac-globulin from the prothrombin during the purification procedure. By replacing the Ac-globulin lost in the purification processes, the rate of thrombin formation is greatly increased and the final thrombin yield is almost doubled. This effect is illustrated in Fig. 1. The curve at the extreme right represents the activation of purified prothrombin alone. By adding increasing amounts of Ac-globulin, the activation rates are increased in proportion. Using a large amount of Ac-globulin shortens the conversion time to 3 minutes. This is comparable to the rate of activation of native bovine prothrombin.

It is apparent that a quantitative relationship exists between the prothrombin activation rate and Ac-globulin activity, provided that other factors which effect prothrombin activation are kept constant; namely, prothrombin concentration, temperature, pH, calcium ions, and thromboplastin. Except for a constant prothrombin concentration, these requirements are fulfilled in the two-stage assay procedure. To control pro-

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* Aided by a grant from the United States Public Health Service.

thrombin concentration a standard purified prothrombin preparation is used.

Standardization of Prothrombin—The most highly purified preparations described by Seegers, Loomis, and Vandenbelt (6) are used. The concentration is adjusted to give 12 second clotting with fibrinogen when completely converted to thrombin in the presence of an excess of Ac-globulin. Beef serum, diluted 800 times, is a suitable source and concentration of Ac-globulin for this purpose. The standard prothrombin is next activated under the same conditions without the addition of serum. A curve similar to the one at the extreme right of Fig. 1 is obtained. This, then, indicates how much Ac-globulin, if any, is present in the standard prothrombin (traces may also be present in the thromboplastin). Now one is prepared to determine the concentration of Ac-globulin in an unknown.

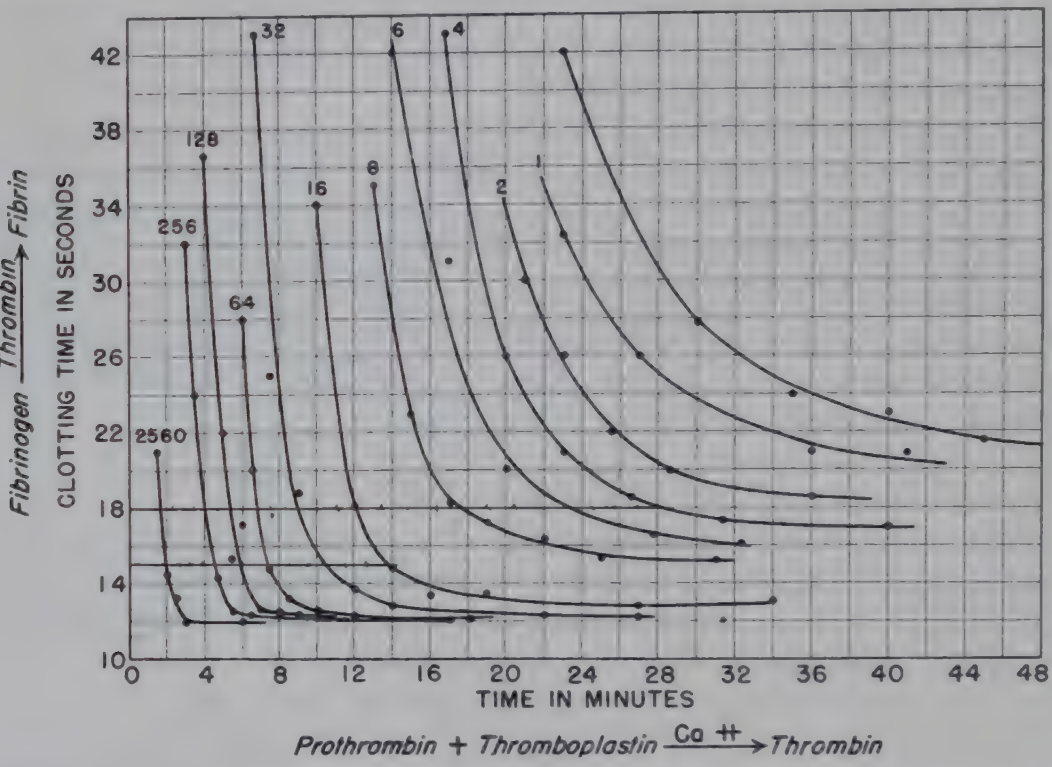

FIG. 1. Acceleration of prothrombin activation with increasing concentrations of Ac-globulin. The concentrations of Ac-globulin are represented by the numbers on the curves. In all instances the prothrombin concentrations were the same. Temperature (28°), calcium, NaCl, thromboplastin, pH, and fibrinogen were constant throughout. The upper right-hand curve was obtained without adding Ac-globulin. The solid lines indicate the clotting time coordinates.

Assay Procedure—The standard prothrombin is mixed with an unknown quantity of Ac-globulin. Data on thrombin formation are again obtained.

The rate of thrombin formation is then plotted in Fig. 1 and an estimate of Ac-globulin activity in the unknown can be calculated. If the curve does not fall between Curves 16 and 128 in Fig. 1, a new dilution of unknown should be made because this is the most accurate working range. When Curve 1 in Fig. 1 is duplicated, the Ac-globulin concentration in the final reaction mixture is, by definition, 1:1000 of a unit per ml. Curve 2560 at the extreme left can be obtained with 2.56 units of Ac-globulin per ml. When the unknown activation curve is definitely located in Fig. 1, the points where it crosses the 18 and 15 second clotting time coordinates (the heavy lines) are used for interpolating the final concentration of Ac-globulin.

If the unknown Ac-globulin sample contains prothrombin, the concentration of the latter must first be determined quantitatively. The standard prothrombin solution must then be adjusted to account for the additional prothrombin which is introduced by the unknown itself.

Analytical Results—When fresh oxalated bovine plasma is used as a source of Ac-globulin, it is found to possess about 150 units per ml. In other words, the concentration of Ac-globulin in plasma is 150,000 times that amount present in the reaction mixture which gave the activation Curve 1, Fig. 1. It is thus seen that the Ac-globulin activity of plasma is very high. By comparison, plasma prothrombin, diluted to the same extent, cannot be detected. The best Ac-globulin preparation which we have made to date possessed a specific activity of 330 units per mg. of dry weight. This was not a pure product but an important calculation can be made. Since bovine plasma contains about 150 units of Ac-globulin per ml., it follows that Ac-globulin constitutes less than 0.7 per cent of the total plasma proteins in bovine plasma and would not be detected by routine electrophoretic analysis.

Properties of Ac-Globulin

Ac-Globulin has properties which make complete chemical separation from prothrombin difficult. Like prothrombin, it is precipitated almost quantitatively from acidified diluted plasma, provided a special anticoagulant (10), low in electrolyte concentration, is used. When the precipitate derived from acidified diluted plasma is redissolved in oxalated saline, the new factor can be adsorbed on magnesium hydroxide. It can then be eluted with carbon dioxide and a relatively stable product is obtained. In contrast to prothrombin it cannot be eluted with phosphate or citrate buffers, because those eluates are quite alkaline, leading to destruction of Ac-globulin activity. Aluminum hydroxide can also be employed for adsorption. Furthermore, it is then possible to elute Ac-globulin with phosphate buffer and obtain a stable product. Ac-Globulin is less soluble in concentrated ammonium sulfate solutions than prothrombin. It is water-soluble but is precipitated from aqueous solutions almost quantitatively at pH 5.4.

Preparation of Ac-Globulin

The following method for purifying Ac-Globulin has been the most successful of many procedures attempted. With this procedure, we have made many preparations of Ac-Globulin of high purity and potency.

Prothrombin product No. 3, described by Seegers, Loomis, and Vandenbelt (6), is first prepared exactly as described. Cool to 0°; with stirring add dropwise an ammonium sulfate solution (saturated at 25°) to 30 per cent of saturation. Strain off the fibrinogen and remove the remaining suspended protein by centrifugation at 0°. Discard the precipitate which is largely inert and add saturated ammonium sulfate dropwise with stirring to 50 per cent of saturation. Let stand at 0° for 20 minutes to permit settling of inorganic salts. The protein precipitate, which contains most of the Ac-globulin, does not settle during this period and it is then removed from the supernatant fluid by centrifugation at 0°. Dissolve the precipitate in 100 ml. of ice-cold water. At 0° add saturated ammonium sulfate dropwise with stirring to 45 per cent of saturation. Discard the supernatant fluid which contains prothrombin in appreciable quantities. Dissolve the precipitate in 10 ml. of ice-cold water and dialyze against repeated changes of ice-cold distilled water at pH 7.0 until the specific resistance is 3000 to 4000 ohms when measured at 5°. The dialysis procedure should not require more than 1½ hours or large losses of Ac-globulin activity will occur. The Ac-Globulin can now be purified by isoelectric fractionation. Adjust the pH to 7.0 and centrifuge at 0°. Discard the precipitate, which contains some Ac-globulin and relatively large amounts of inert protein. Acidify the supernatant fluid to pH 5.4 by adding 0.1 N hydrochloric acid with stirring. Remove the precipitate by centrifugation at 0°. The supernatant fluid contains very little Ac-Globulin but relatively large amounts of prothrombin. The precipitate represents the final purified product. It is dissolved in 10 ml. of cold water with addition of 0.1 N sodium hydroxide to pH 7.0, care being taken to avoid local excess of alkali by stirring. The product can be dried from the frozen state without loss of activity.

Electrophoresis of Purified Ac-Globulin

The best Ac-globulin preparations we have to date are not stable for sufficient time to maintain full activity during electrophoretic examination in the Tiselius apparatus. It is nevertheless possible to gain much information from such studies. A preparation possessing 3500 units per mg. of tyrosine was placed in phosphate buffer of pH 7.4 and 0.2 ionic strength. This preparation contained about 5 per cent prothrombin as impurity.

There is only slight evidence of this rapidly moving prothrombin in the pattern shown in Fig. 2. Two major components predominate. One of these moved with a mobility of -2.96×10^{-5}, which corresponds to β-globulin. The other component gave the mobility value, -4.73×10^{-5}. It very likely represents Ac-globulin. This mobility is thus greater than that for α-, β-, or γ-globulin. If we assume that this rapid moving component represents Ac-globulin, then our product is somewhere in the neighborhood of 50 per cent pure. This would mean that Ac-globulin comprises from 0.4 to 0.5 per cent of the total protein concentration in bovine plasma (*cf.* discussion above).

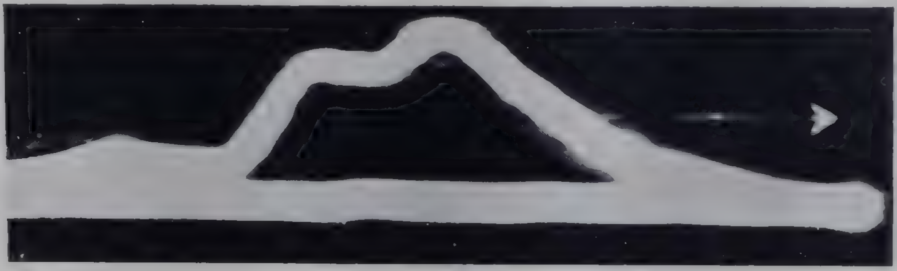

FIG. 2. Electrophoretic pattern of purified Ac-globulin at pH 7.4; phosphate buffer; ascending boundary at 118 minutes. The beginning boundary is at the left.

TABLE I

Stability of Ac-Globulin in Oxalated Bovine Plasma at 5°

Days of storage	Ac-Globulin				
	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5
	units per ml.	*units per ml.*	*units per ml.*	*units per ml.*	*units per ml.*
0	130	140	140	140	140
5	138	120	110	115	130
10	115	165	100	120	105
20	80	95	100	120	105
40	110	70	90	95	95
60	80	70	90	95	85

Stability of Ac-Globulin

A study has been made of the stability of Ac-globulin in storage plasma. At the slaughter-house 7 parts of cow blood were mixed with 1 part of 1.85 per cent potassium oxalate. The oxalated blood was cooled, taken to the laboratory, and centrifuged at 0°. Plasma obtained in this manner from five cows was stored at 5° and Ac-globulin analyses were made at regular intervals. Table I contains a summary of the results obtained over a period of 60 days. It is clearly shown that Ac-globulin is quite stable in oxalated bovine plasma stored under these conditions.

In contrast aqueous solutions of purified Ac-globulin lose activity rapidly. It, therefore, was of interest to study inactivation at various temperatures. A product with a specific activity of 1000 units per mg. of tyrosine was placed in neutral saline solution and aliquot portions were heated at various temperatures for 30 minutes. The results are presented in Fig. 3 and are compared with prothrombin inactivation curves published by Seegers (11) in 1940. His inactivation curves were obtained by exactly the same technique. He pointed out that heat-treated prothrombin is less reactive to thromboplastin than prothrombin not heated (11). By allowing more time for thromboplastin to act he minimized the effect of Ac-globulin destruction. This compensation for Ac-globulin destruction is indicated by Area Y in Fig. 3.

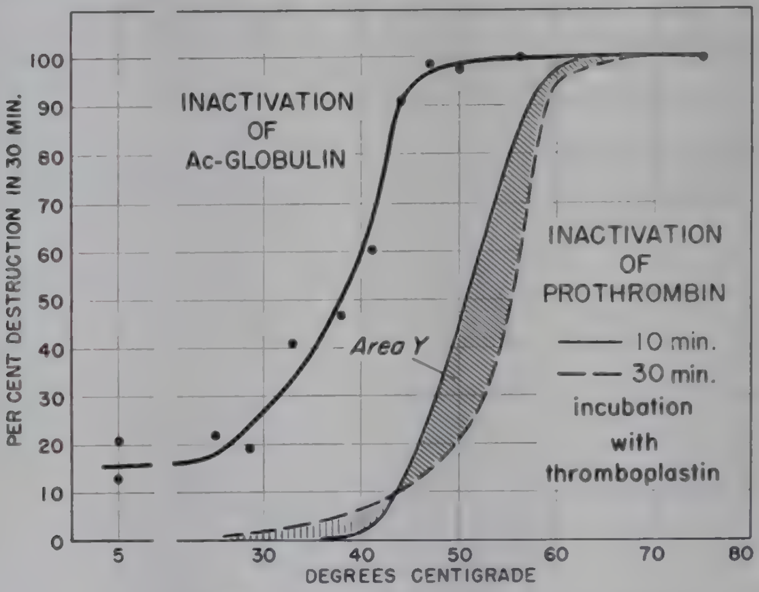

FIG. 3. Inactivation of purified Ac-globulin by heating to various temperatures for 30 minutes in neutral saline solution. The curves for prothrombin inactivation under similar conditions are reproduced from previously published data (11). Area Y represents Ac-globulin destruction.

DISCUSSION

We believe that Ac-globulin is identical with the accelerator factor referred to in a preliminary report by Fantl and Nance (1) and with factor V of Owren (2, 3). Our preliminary reports (4, 5) do not refer to Owren's work because we were not aware of his contribution which he indicates was first announced in 1944. Due to the War, this announcement apparently did not come to the attention of Fantl and Nance.

The question may be asked whether Ac-globulin could not be regarded as the hypothetical prothrombin A of Quick (12). In agreement with Fantl and Nance (1) we do not consider that conclusion possible for several reasons. Ac-Globulin is quite stable in oxalated bovine plasma and is adsorbed on magnesium hydroxide and aluminum hydroxide. Quick finds the opposite for his factor and in addition he has not described prothrombin A as an accelerator of prothrombin activation (12–16). The phenomena which Quick has described as indicating two prothrombins (12) have been found to be largely due to the mixing of plasmas of different species and to the effects of storage on plasma fibrinogen reactivity (17).

SUMMARY

Plasma contains a factor, Ac-globulin, which accelerates the activation of prothrombin in the presence of thromboplastin and calcium ions. It has been obtained in concentrated form. This globulin is less soluble in concentrated ammonium sulfate solutions than prothrombin. It is far more sensitive to heat than prothrombin. It is adsorbed on magnesium hydroxide and aluminum hydroxide. It is stable in storage plasma. Its concentration in bovine plasma is less than 0.7 per cent of the total plasma proteins. A quantitative method for the determination of Ac-globulin is described. With the aid of reasonable assumptions it has been shown that the electrophoretic mobility of Ac-globulin is -4.73×10^{-5} in phosphate buffer of pH 7.4.

We wish to thank Mr. Carl Hyde for technical assistance, Dr. J. M. Vandenbelt for placing electrophoretic data at our disposal, and Parke, Davis and Company for large quantities of plasma and for funds for research in Physiology.

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THE ENZYMATIC EXTRACTION OF RIBOFLAVIN FROM PORK FOR THE FLUOROMETRIC DETERMINATION*

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The literature contains a number of references to apparent increases in riboflavin as measured by either microbiological or fluorometric procedures when various raw foods are cooked or processed. Rice *et al.* (5), using a microbiological method, found a large increase in the riboflavin content of pork stored at 4° and at room temperature for some days. They attributed the increase to bacterial synthesis of riboflavin, since visible spoilage occurred in all samples showing the increase. Hinman *et al.* (3), in an exhaustive study employing both microbiological and a variety of fluorometric procedures, found a definite increase in riboflavin on braising of beef. Gleim *et al.* (2), using a fluorometric method, report retentions around 100 per cent when carrots were boiled, but apparent retentions of approximately 130 per cent when the carrots were held 1½ to 3 hours at 60–70°. Richardson *et al.* (6) obtained apparent retentions in excess of 200 per cent on canning beef (fluorometric procedure), *but when the experiments were repeated by the rat growth method, the raw samples of meat showed a riboflavin content almost 3 times as high as was indicated by fluorometric analysis, and a slight loss of riboflavin occurred on canning.*

Similar large apparent increases have been noted frequently in this laboratory during recent months with fluorometric procedures. Murray[1] found retentions up to 150 per cent when dried peas were soaked and cooked. McGregor[2] found increased amounts of riboflavin in lamb after 6 months of freezing storage and still higher amounts after 9 months, as well as large increases on canning. Watts and Peng[3] obtained apparent retentions which sometimes exceeded 300 per cent on the dry, fat-free basis when

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[1] Murray, H., unpublished.

[2] McGregor, M., unpublished.

[3] Watts, B. M., and Peng, D. H., unpublished.

pork ribs and loins were held in freezing storage for 9 months, then roasted. The increases were considerably greater in pork cooked after freezing than in paired cuts cooked before freezing. There were no visible signs of spoilage and the pork rated good to excellent in palatability tests.

Many of the microbiological and fluorometric procedures, as currently used, depend upon a preliminary heating (boiling or autoclaving) in acid solution, followed by an enzymatic digestion with clarase or other commercial enzyme preparations to free riboflavin from combinations in the tissues. The kinds of food processing which have resulted in an apparent increase in riboflavin have in common the fact that the raw tissue is exposed for a period of time to its own enzymes before these enzymes are destroyed by heating at the pH of the original tissue. During braising of beef roasts or canning of meat, the temperature within the meat or can rises slowly; enzymatic activity might be expected to be accelerated at least during the preliminary stages of such cooking.

The evidence points to the possibility of a riboflavin combination in tissues which is only partially released by clarase and other commonly used commercial enzyme preparations acting on heat-denatured tissues, but which may be released by enzymes within the tissue itself. The present study was designed to test this hypothesis by comparing riboflavin values obtained on the raw pork by a widely used fluorometric procedure with values obtained on the same pork after a preliminary autodigestion.

In view of the difference in time of extraction recommended by Connor and Straub (1) and Peterson, Brady, and Shaw (4), it also seemed advisable to investigate further the time factor in relation to the period of incubation.

Procedure

The rib eye (longissimus dorsi) muscle from fresh pork was used for all experiments. Meat was obtained from a local butcher. The muscle was trimmed, put through a meat grinder, and thoroughly mixed. Samples not used immediately were heat-sealed in pliofilm and frozen at −17° until ready for use.

The fluorometric procedure of Peterson, Brady, and Shaw (4) was used with a slight modification of the extraction process as follows.

Duplicate samples (45 gm.) of the ground meat were weighed and blended for 2 minutes in a Waring blendor with 105 ml. of 0.04 N sulfuric acid. 35 gm. aliquots of this total suspension (10.5 gm. of meat) were then transferred to 100 ml. volumetric flasks. To each flask were added an additional 35 ml. of the acid. The flasks were then heated in a boiling water bath for 1 hour with frequent agitation and cooled to below 50°. To each flask was added 0.5 gm. of clarase and the pH adjusted to 4.5 with 0.5 N sodium acetate, with bromocresol green as an outside indicator. The mixture was incubated at 45° overnight (15 to 16 hours), cooled, made up to 100 ml. with distilled water, mixed, and filtered. If necessary further dilution to an estimated riboflavin concentration of 0.5 to 2.0 γ per ml. was made. The clear filtrate was used for fluorometric assay as described by Peterson *et al.* (4). All operations were carried out in semidarkness.

Two variations of the extraction procedure were tried in order to investigate the effect of preliminary digestion by enzymes in the meat. In the first variation, the meat was allowed to digest without clarase, then heated with acid, and finally digested with clarase. In a second variation, clarase was added and allowed to act with the meat enzymes. The sample was then heated with acid and analyzed without any further digestion of the heated material. Details of the procedures were as follows.

Variation 1—45 gm. samples of meat were blended with 55 ml. of distilled water and 10 ml. of chloroform to prevent bacterial growth. The blendor jar was covered with aluminum foil to prevent evaporation and the blended samples were incubated at 45° for 16 hours. At the end of the incubation period 40 ml. of 0.1 N sulfuric acid were added (with the same total acid concentration as in the standard sample) and the sample again blended. Three 35 gm. aliquots representing in this case 10.5 gm. of meat were then transferred to 100 ml. volumetric flasks and the analysis completed as in the standard method.

Variation 2—45 gm. samples of meat were blended with 105 ml. of 0.04 N H_2SO_4, as in the standard method. 35 gm. aliquots of the total suspension were transferred to 100 ml. volumetric flasks. The pH, in all cases higher than 4.5, was adjusted to 4.5 by the addition of 0.04 N sulfuric acid. 0.5 gm. of clarase and 3 ml. of chloroform were added to each flask, mixed, and incubated for 16 hours. At the end of the incubation period 0.04 N H_2SO_4 was added to make a total of 35 ml. of added acid. The digest was then heated in the boiling water bath for 1 hour, cooled, pH again adjusted to 4.5 with 0.5 N sodium acetate, and made to volume with distilled water. Again the analysis was completed as in the standard method. Since most of the chloroform was evaporated during the heating period, no correction was made for volume occupied by the chloroform in Variations 1 or 2.

Periods of incubation time ranging from 6 to 72 hours were investigated with both the standard method and Variation 2.

Results

Extraction of riboflavin from raw pork is increased by an incubation period preliminary to the heat treatment (Table I). With autodigestion plus clarase the riboflavin was markedly increased, as compared with the standard method. This is true whether the enzyme preparation was added to the autodigest after the heat treatment (Variation 1) or at the beginning of the preliminary incubation (Variation 2). The somewhat higher values obtained in Variation 2 as compared to Variation 1 may be due to any one of several causes; *i.e.*, (1) to the fact that autodigestion was carried out at a lower pH (4.5 in Variation 2 as compared with the original pH of the meat, approximately 6.0 in Variation 1), (2) to a greater effect of clarase on raw than on heated meat tissues, or (3) to liberation of increased amounts of riboflavin from clarase by enzymes within the meat.

However, incubation preliminary to heat treatment failed to increase the riboflavin extraction from roasted pork (Sample VI, Table I) which had been stored frozen for 1 year. Both the standard method of extraction and Variation 2 resulted in approximately the same amount of riboflavin. This would indicate that following the denaturation of the enzymes within the meat by heat, the liberation of riboflavin by clarase is retarded.

TABLE I

Effect of Autolysis on Riboflavin Obtained from Raw Pork

Pork sample No.	Clarase sample No.	Riboflavin, γ per 100 gm. pork		
		Standard method	Variation 1	Variation 2
I	1	58	107	132
I	2	112		
II	2	81		129
IV	2	78		120
IV*	2	89		139
VI†	1	107		102

* After storage at −17° for 1 week.

† Roasted, frozen for 1 year.

The first sample of clarase used gave very low values for riboflavin by the standard method. This lot was 3 years old. In addition it had a very high enzyme blank (18.9 γ per gm. of enzyme). A new purchase of clarase containing less riboflavin (5.5 γ per gm. of enzyme) released almost twice as much riboflavin from the same sample of meat by the standard method. This newer product was used in all later experiments.

Since Variation 2 resulted in the highest riboflavin values, it was used to compare with the standard method in respect to variable extraction periods. Contrary to Peterson *et al.* (4) increased readings for riboflavin were obtained with increasing time (Table II) at least up to 60 hours. All but one series showed this increase up to 72 hours, the maximum length of time tried. These increased values were obtained with both methods of extraction, although the amount of riboflavin extracted from the heat-treated tissue was always less than with the preliminary incubation. At the end of 72 hours the riboflavin extracted by the predigestion procedure was approximately 3 times the amount originally found by the standard procedure.

Although there was no apparent indication of spoilage, the efficiency of chloroform as a preservative was naturally questioned in view of these results. Because of its higher boiling point, toluene was chosen as an added preservative. To each 35 gm. aliquot, 4 ml. of toluene were added to the chloroform-meat suspension before incubation. The added preservative did not prevent these increased riboflavin values (Table II). Again, Variation 2 resulted in higher values than in the standard method.

As a further check on the efficacy of toluene as a preservative the riboflavin in meat Sample V was extracted as in the standard method, with and without added toluene (Table II). At 16 and 72 hours, the riboflavin values obtained by a chloroform-toluene suspension were substantially the same as those secured from the chloroform suspension alone. Since the added preservative action afforded by toluene did not prevent the increased riboflavin values with time, it is assumed that the increase is caused by enzymatic release of the riboflavin rather than bacterial synthesis.

TABLE II

Effect of Digestion Time on Extraction of Riboflavin from Raw Pork

Pork sample No.	Extraction procedure	Preservative used	Riboflavin at various digestion intervals, γ per 100 gm. fresh raw pork						
			6 hrs.	16 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.
III	Variation 2	Chloroform	78	126	149	192			
IV	Standard	"		78		128	134	165	167
	Variation 2	"		120		166	167	201	278
IV*	Standard	" and toluene		89		140	139		155
	Variation 2	" " "		139		176	181		236
V	Standard	"		107					146
	"	" and toluene		107					155

* After storage at −17° for 1 week.

DISCUSSION

The results demonstrate that present widely used methods of extraction for the fluorometric assay of riboflavin give a variable fraction of the total riboflavin present in meat. Enzymes within the meat itself bring about a progressive increase in the riboflavin liberated if opportunity is allowed for autodigestion in the presence of preservatives. This enzymatic liberation of riboflavin probably continues at the low temperatures of freezing storage and may be greatly accelerated at higher temperatures encountered in the preliminary stages of cooking.

Microbiological riboflavin assays are also carried out on filtrates of meat extracted by methods similar to those used for the fluorometric procedure. Autoclaving in acid solution or treatment with commercial enzymes is relied upon for extraction, and detailed comparisons on the amount of riboflavin freed by these procedures are lacking (7).

On the other hand, it might be expected that the biological assay would more nearly approximate the total riboflavin present, since animal tissues apparently contain enzymes capable of releasing the bound form of the vitamin. The only comparison, of which the authors are aware, of fluorometric and biological assays on raw and cooked meat (6) seems to support this view.

More work on extraction procedures seems to be imperative before reliance can be placed on figures for the riboflavin content of meats or riboflavin losses during meat processing.

Further investigation of the extraction and determination of riboflavin in plant and animal tissue by both chemical and biological methods are being continued in this laboratory.

SUMMARY

Data have been presented which indicate that extraction of riboflavin from pork muscle is increased by an incubation period at 45° preliminary to the denaturation of the tissue by heat at 100°. The presence of clarase during this preliminary incubation extraction facilitates this apparent autolysis and increases the riboflavin values.

The adequacy of the generally accepted 24 hour extraction period is questioned. Increasing riboflavin values were obtained up to 72 hours of incubation, the maximum length of time tried in the presence of preservatives.

Incomplete extraction of potentially active riboflavin may account for reported increased yields after processing or storage.

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FRACTIONATION OF SERUM WITH METHANOL AND ULTRAFILTRATION

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A method for determining the albumin-globulin ratio in serum by precipitation with methanol at 0° has been described by Pillemer and Hutchinson (1). The authors compared the ratios so determined with values found by electrophoretic analysis and reported excellent agreement. They employed the method only for analytical purposes. This paper describes the use of Pillemer and Hutchinson's method, without essential modification, in conjunction with ultrafiltration (2) for the isolation of albumin and globulin fractions, both from human sera and from sera of normal and immunized animals.

Method

The method of Pillemer and Hutchinson requires that all operations be carried out between 0–1°. 2 ml. of sera and 1 ml. of sodium acetate buffer, pH 4.0, are well mixed and cooled to about 4°. 7 ml. of a diluted methyl alcohol solution are cooled to 0° or slightly lower. The two solutions are then well mixed and maintained at approximately 0° for 30 minutes. This gives a final alcohol concentration of about 42.5 per cent and a pH of between 6.7 and 6.9. Under these conditions the globulins are quantitatively precipitated, while the albumin remains in solution.

EXPERIMENTAL

The sera used included a pool of rabbit sera prepared approximately 1 year previously, a pool of fresh guinea pig sera, a pool of fresh human sera that did not react in serologic tests for syphilis, horse serum from an individual bleeding 7 months old, and an antipneumococcus type 2 rabbit serum from a pool 4 years old. 50 ml. of sera were used in each experiment.

A methanol solution, 60 per cent by volume at room temperature, was adopted as the standard precipitating solution. The amount of buffer in the several experiments was varied to obtain a final pH of 6.7 to 6.9. Less buffer was required with rabbit and guinea pig sera than with human serum; more buffer, with horse serum.

The precipitated globulins were filtered by suction in the cold room (8°) on a Büchner funnel. A No. 50 Whatman filter paper coated with 1 gm. of Hyflo Filter-Cel moistened with 40 per cent methanol was employed for retention of the precipitate. Since filtration time was only about 30 minutes, the rise in temperature from 0° was not harmful. After removal from the filter paper, the globulins plus Filter-Cel were dried overnight in a desiccator containing sulfuric acid. The paper, retaining some of the globulin, was dried simultaneously in the same desiccator. The dried globulin was dissolved in 1 per cent sodium chloride, adjusted to pH 7.4 if necessary, centrifuged to remove Filter-Cel and shredded filter paper, and restored to the original volume. The albumin-methanol filtrate was diluted 1:2 with distilled water to avoid undue swelling of the membrane and was adjusted to pH 7.4 to 7.6 to suppress adsorption upon the membrane (3).

The albumin-methanol solution was concentrated to about 25 ml. by filtration upon a 9.5 per cent parlodion nitrocellulose membrane. The albumin solution was then diluted about 10-fold with sterile distilled water and once more concentrated by filtration to about 25 ml. This was repeated several times to remove the methanol, after which the albumin was removed from the ultrafilter and diluted to its original volume with water. The results are recorded in Table I.

TABLE I

Methanol Fractionation of Sera

Species	pH	Protein		Albumin		Globulin		Albumin-globulin ratio		Non-protein N
		Kjeldahl	$CuSO_4$	Kjeldahl	$CuSO_4$	Kjeldahl	$CuSO_4$	Kjeldahl	$CuSO_4$	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			*mg. per ml.*
Rabbit	7.6	6.15	6.19	4.40	4.39	1.53	1.66	2.9	2.6	0.36
Guinea pig	8.15	4.84	4.90	3.51	3.46	1.25	1.30	2.8	2.7	0.41
Human	8.4	7.40	7.36	4.58	4.61	2.72	2.81	1.7	1.6	0.30
Horse	7.6	7.10	7.06	3.91	3.89	3.15	2.99	1.2	1.3	0.23
Rabbit*	8.0	9.12	9.18	2.84	2.99	5.47	5.40	0.5	0.6	0.76

* Antipneumococcus type 2 rabbit serum.

The nitrogen content of the various sera and fractions was determined by the usual micro-Kjeldahl procedure. The non-protein nitrogen was determined after removal of the protein with trichloroacetic acid. The factor 6.25 was used to calculate protein from protein nitrogen.

To furnish an alternative estimation of the protein content of the various solutions, the copper sulfate density method of Phillips and his coworkers (4) was used. Their study was concerned with plasma proteins, but the serum density correspondingly indicates the serum proteins. Since the original article appeared, the authors have modified the equation for normal human plasma, which now appears as

$$P = 389.6\ (G_p - 1.0079) \tag{1}$$

where P is the plasma protein concentration in gm. per 100 ml.; G_p, the density of the plasma; 1.0079, the density of the ultrafiltrate of the plasma which consists of water, salts, etc.; and 389.6 is an empirical coefficient. The equation previously employed was

$$P = 360\ (G_p - 1.0070) \tag{2}$$

The copper sulfate density determinations of the sera and of the albumin and globulin preparations are recorded in Table II. The figures in the fourth decimal place are interpolated values. In the determination of protein in normal human sera when equation (1) was used, close agreement was obtained between results by the Kjeldahl and copper sulfate density methods. However, for other normal animal sera and for all these fractions, including those from the immune sera, the use of equation (2) was much more satisfactory. In calculating the protein content of the globulin fractions, 1.0070 was used, as indicated, inasmuch as the density of 1 per cent sodium chloride is 1.0070 at 25°/25°. Since the albumin fractions are washed free from salts and methanol, 1.0000 (by definition the density of water at 25°/25°) is employed instead in the equation.

TABLE II

Density of Sera and Fractions

Species	Sera	Albumin	Globulin
Rabbit	1.0242	1.0122	1.0116
Guinea pig	1.0206	1.0096	1.0106
Human	1.0268	1.0128	1.0148
Horse	1.0266	1.0108	1.0153
Rabbit*	1.0340	1.0083	1.0220

* Antipneumococcus type 2 rabbit serum

DISCUSSION

The results obtained in the fractionation of rabbit sera as shown in Table I agree fairly well with those obtained by Bjørneboe and Gormsen (5). They found the average protein content of seventeen normal rabbit sera to be 6.03 per cent, the globulin content 1.23 per cent, and the albumin 4.81 per cent. The average globulin content of the sera of young rabbits was 1.42 per cent, but old rabbits averaged 0.95 per cent. A globulin content in excess of 2 per cent was considered abnormal.

The results with human sera are similar to those obtained by Pillemer and Hutchinson. Fractionation of normal horse serum yielded results resembling those of Butler and Montgomery (6), who fractionated normal horse sera by both the $(NH_4)_2SO_4$ and Na_2SO_4 methods and estimated the albumin-globulin ratio to be 1.24 by the former method and 1.10 by the latter.

When immune rabbit serum was ultrafiltered through a 9.5 per cent parlodion membrane, the protein-free ultrafiltrate contained 0.4 per cent chlorides and had a density of 1.0085. When this density was used in place of 1.0070 in equation (2), the protein content so calculated was approximately the same as that obtained by the Kjeldahl method. The pool of 4 year-old sera gave indications of amino acid inhibition of copper proteinate formation (7).

As a check upon the efficacy of the fractionation method with immune sera, Heidelberger's (8) method for precipitation of antibody nitrogen by the type-specific carbohydrate was used.[1] From the unconcentrated serum 4.68 mg. of antibody nitrogen per ml. were precipitated, and from the globulin diluted to volume, 4.21 mg. per ml. No precipitate resulted when the type-specific carbohydrate was added to the albumin fraction. These results indicate satisfactory recovery and considerable purification of the globulin and that the globulin was not significantly denatured by the methanol precipitation. Accordingly the procedure should be a useful preliminary step in the study of such antibodies.

SUMMARY

The preparation of serum albumin and globulin fractions by a combination of methanol precipitation and ultrafiltration is described. The method is applicable to various normal and immune animal sera as well as to human serum.

The protein content of various sera and of albumin and globulin solutions derived from them was precisely estimated by the copper sulfate density method.

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[1] These tests were made by Miss Gretchen Sickles.

PROTOPORPHYRIN 9 AS A PRECURSOR OF CHLOROPHYLL*

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Chlorella vulgaris is an ovoid plant cell of 3 to 10 μ diameter which possesses a single cup-shaped chloroplast containing the chlorophyll and carotenoids. This cell normally synthesizes these pigments in the dark when grown on a glucose-inorganic salt medium.

In an attempt to analyze the steps in the synthesis of these pigments, mutants of the *Chlorella* cells were prepared by irradiation with x-rays. It was hoped that in this manner some of the intermediate stages in the synthesis might be prevented and that intermediate products might accumulate. This paper is concerned with the isolation and characterization of a brownish red fluorescent pigment which appeared in one of the mutants.

EXPERIMENTAL

Origin of Mutant Chlorella W_5 Brown—Chlorella vulgaris cells, grown on the inorganic medium of Craig and Trelease (2) in the light, were concentrated by centrifugation, suspended in a small drop of the same medium in a thin walled Carrel flask, and exposed to x-rays from a 180 kilovolt tungsten target tube at a rate of 2050 R.P.M. for 10 to 20 minutes, the tube radiation half value layer being 0.19 mm. of Cu.

The treated cells were then diluted and from that time on were grown in the dark on a solid medium consisting of 3 per cent agar, 0.7 per cent glucose, and the inorganic salts. Colorless, pale green, and yellow colonies arose. A mutant, W_5, with which we shall be primarily concerned here, was isolated early in 1946 as a colorless colony. It has a tendency to give rise to pale green colonies. The pale green colonies may give rise to colorless colonies again. On solid media, both the pale green and colorless cells have given rise at times to brown colonies. Colonies arising from single cells derived from these brown areas have been further selected for their tendency to turn brown.

When the brown cell, W_5 brown, is grown in a liquid medium, even with continual aeration, the developing cells appear only faintly brown in a thick layer. If grown in test-tubes on agar slants or in Petri dishes, the brown color develops within 1 to a few weeks. For large flasks and rapid development of the brown color the following procedure has been used. An inoculum, taken from brown cells on solid medium or from the almost colorless cells grown on liquid medium, was sown on solid medium. At 25° growth was maximum on the 4th day, but the cells were colorless; at this time the flasks were tilted slightly to drain away excess surface liquid, and within the next 2 to 3 days the cells became brownish. If the liquid was not drained away some 4 to 6 days after inoculation, only a slight browning resulted within the next few days The impression was gained that the brown pigment develops most rapidly in the resting stage at the end of the logarithmic period of growth.

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* This is the third in a series of studies on porphyrins and related compounds. For the second paper see Gilder and Granick (1).

Under these conditions of growth the cells often were enlarged to 2 to 3 times their normal diameter, containing some five to twenty large starch grains which appeared to be localized in a vacuole making up most of the cell volume; a thin crescent-shaped area of cytoplasm containing the granular or reticulated brown material lay on one side of the cell. These cells were easily broken by merely shaking in distilled water. Small cells were seldom observed to contain this brown material.

Properties of Brown Material—In the hand spectroscope a suspension of the brown cells revealed three bands with maxima at 530, 590, and 645 mμ. No band at 505 mμ was visible. By washing the cells with water and centrifuging at 500 × g, a brownish cloudy supernatant solution was obtained whose absorption, when measured with the Beckman spectrophotometer, revealed the same bands (Fig. 1, Curve A). When this brown pigment was extracted, dissolved in dilute alkaline ethanol solvent, and brought to the same volume as the original suspension, a curve (Fig. 1, Curve B) typical of protoporphyrin was obtained.

The brown color of the cell suspension was found to correspond in spectral properties to a colloidal suspension of protoporphyrin. When crystalline protoporphyrin was dissolved in 0.02 N potassium hydroxide containing 50 per cent by volume of ethyl alcohol, a pink solution of the monomeric form of protoporphyrin was obtained (Fig. 2, Curve A). When this solution was neutralized with acetic acid, the protoporphyrin became colloidally polymerized and its absorption bands changed (Fig. 2, Curve B); the solution turned brownish red in color, the 505 mμ band vanished, and new band maxima arose, corresponding to those observed in the brown *Chlorella* cells. Depending on the degree of polymerization, various intermediate types of absorption spectra between those of the monomer and the high polymer could be obtained.

The brown pigment was most readily released from the cells by wetting the packed cells with a mixture of glacial acetic acid containing a small amount of HCl and extracting with an equal volume of ether. From neutral or alkaline solutions the pigment was only extracted into organic solvents with difficulty, although the small amounts that were extracted showed the typical protoporphyrin spectrum. Advantage was taken of this relative insolubility of the pigment at neutral pH to get rid of fatty materials of the cells by a preliminary extraction with ether and alcohol before extracting the main bulk of the pigment.

Isolation of Pigment from Cells—About 50 cc. of packed cells were first extracted with 150 cc. of ethyl alcohol and then with 150 cc. of 3:1 ether-absolute alcohol to get rid of fatty materials. This solution, which was discarded, was faintly pink, showing the typical bands of protoporphyrin. The cells were next stirred with 50 cc. of acetic acid-HCl (99 parts of acetic acid + 1 part of 6 N HCl), and then 100 cc. of ether were added. The suspension was centrifuged and the supernatant containing the acid form of protoporphyrin was decanted. The extraction was repeated twice more. The combined extracts in a separatory funnel were treated with aqueous 2 N potassium acetate to convert the pigment to the neutral form, and the acetic acid was removed from the ether layer with water.

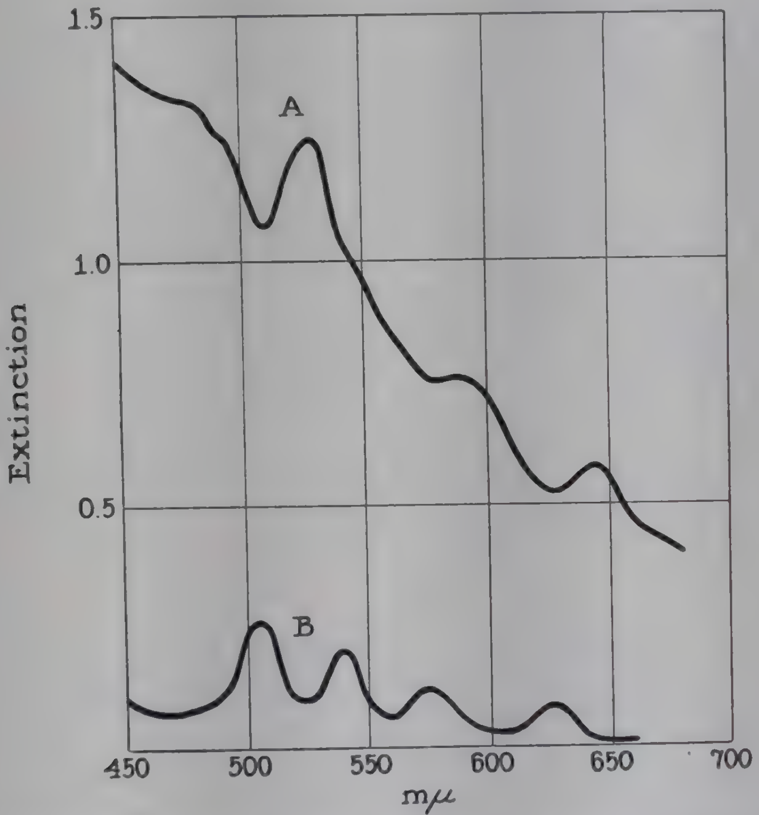


FIG. 1. Curve A, absorption of an aliquot of brown pigment obtained from the W_5 brown cells as a cloudy suspension in water; Curve B, absorption after extraction and dilution in 0.02 N KOH-50 per cent ethanol solvent to the same volume as the original aliquot.

Identification of Pigment As Protoporphyrin—An aliquot of the ether solution was measured spectrophotometrically. The extinction values so obtained were plotted on a curve of known protoporphyrin; one point was multiplied by a factor (*i.e.* 505 mμ). All other points fell on the curve. Within experimental error, the position and height of the band maxima of the porphyrin from *Chlorella* were identical with those from crystalline protoporphyrin.

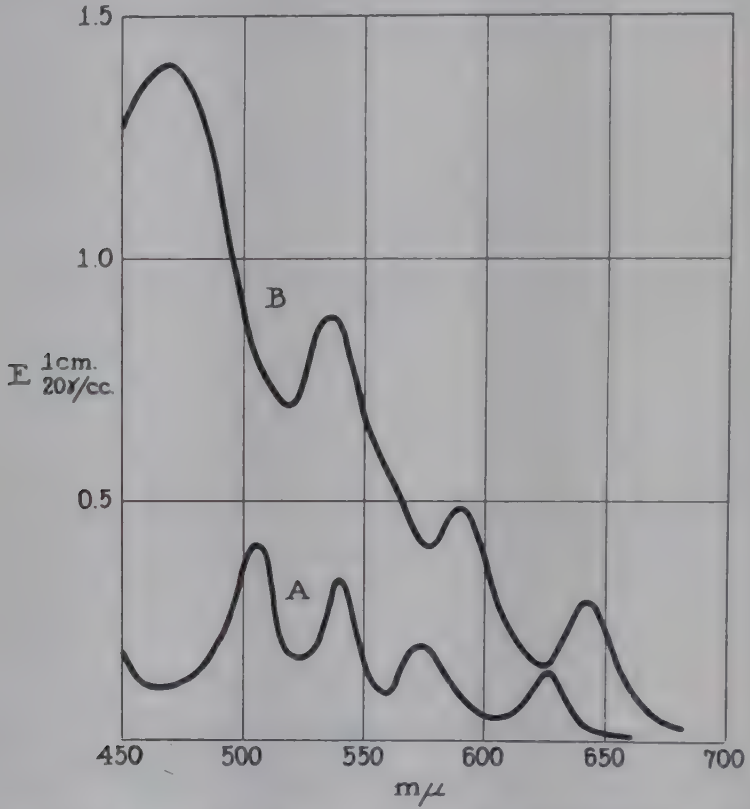


FIG. 2. Extinction E is for a 1 cm. light path, the concentration of protoporphyrin being 20 γ per cc. Curve A represents extinction of a pink protoporphyrin solution dissolved in 0.02 N KOH-50 per cent ethanol; Curve B extinction of 5 cc. of the above protoporphyrin solution to which had been added 0.02 cc. of glacial acetic acid, esulting in a brownish red colloidal solution.

The HCl number of the porphyrin was also determined on another aliquot by successively extracting the pigment into increasingly higher concentrations of aqueous HCl; it was found to be identical with that of protoporphyrin. No porphyrin other than protoporphyrin was detected in the aqueous extracts. A protoporphyrin containing only one vinyl group might have been expected to be extracted from the ether with an HCl solution of from 0.1 to 0.3 N HCl; a porphyrin with such a property was not detected. The main ether solution was extracted with 2 N HCl. (Only a faint residue in the ether remained, the concentration of which was too low to be worked up.)

Further support for the identification of the brown pigment as protoporphyrin was obtained by biological tests. *Hemophilus influenzae* Turner requires iron protoporphyrin or protoporphyrin for growth (1). It may, however, grow when furnished with iron porphyrins which lack vinyl groups. When protoporphyrin was replaced by a suspension of W_5 brown *Chlorella* cells, definite growth of the influenza organism was observed. No support of growth of the influenza organism could be obtained with a suspension of normal green *Chlorella* cells or a suspension of yellow mutant *Chlorella* cells to replace protoporphyrin. This influenza organism has the further agreeable property of reducing nitrate to nitrite only in the presence of vinyl-containing porphyrins, *i.e.* protoporphyrin or iron protoporphyrin (1). No other porphyrins or iron porphyrins have this effect. Quantitative determinations were made of the growth of this organism and its nitrate-reducing ability on protoporphyrin 9 and on the compound isolated from *Chlorella*. For both compounds at a concentration of 0.01 γ of compound per cc. of medium there was a trace of growth and no nitrate reduction. At 0.02 γ per cc. growth was maximum and nitrite formation was maximum for both compounds, further confirming the idea that the compound from *Chlorella* is a protoporphyrin. (Because no isomeric protoporphyrins have been available for determining the specificity of the biological test, it cannot be decided from this experiment that protoporphyrin of *Chlorella* is isomerically identical with that derived from blood heme.)

Identification of Protoporphyrin As Isomer 9—One-fifth of the total HCl extract from the ether was esterified with methyl alcoholic HCl. This crystalline compound had a melting point of 223°, and on recrystallization the melting point was 225°. Too little remained for further recrystallization. A preparation of protoporphyrin 9 dimethyl ester prepared from beef hemoglobin had a melting point of 228°. The literature reports a melting point of 230° for protoporphyrin 9 dimethyl ester. Considering the effect of slight impurities and that the melting points were determined on crystals under the microscope, the melting point of the protoporphyrin dimethyl ester derived from the *Chlorella* cells supports the idea that the protoporphyrin is probably isomer 9.

The accepted method of identifying the isomers is to convert the protoporphyrin to mesoporphyrin dimethyl ester, the melting point of whose isomers are known. The main portion of the HCl extract containing the protoporphyrin was converted to the meso ester in the following manner: The HCl solution (about 50 cc.) was neutralized with 2 M potassium acetate, and the flocculation aided with 0.2 gm. of sodium tartrate. The precipitate was filtered onto a small asbestos mat, washed once rapidly with absolute alcohol, and then dried in a vacuum desiccator.

Conversion to mesoporphyrin was brought about by treating the asbestos mat containing the protoporphyrin with 2.5 cc. of 99 per cent formic acid plus 3 mg. of colloidal palladium (18.2 per cent Pd). This suspension was warmed to 50° and H_2 bubbled in for 10 minutes. The solution was then filtered by suction, and the filter washed with a few drops of formic acid and then with water. The filtrate was transferred to a separatory funnel, diluted with 10 cc. of water, the formic acid neutralized with 2 M potassium acetate, and the porphyrin extracted into the ether. The ether layer was washed several times with water. It was extracted with 0.2 N HCl and then with 1 N HCl. (No evidence for a porphyrin other than mesoporphyrin was observed spectroscopically in these extracts.) The HCl extracts were combined, filtered, reextracted into the ether, and the ether evaporated to dryness.

The resulting dry mesoporphyrin was esterified overnight at room temperature with 15 cc. of dry methanol containing 5 per cent by weight of HCl. The esterified mesoporphyrin was then extracted into 10 cc. of $CHCl_3$. The $CHCl_3$ solution was washed with water and finally with 0.02 N NH_4OH to convert the porphyrin to the neutral form. The $CHCl_3$ was dried with anhydrous sodium acetate, filtered, and evaporated to dryness, and the residue was crystallized from $CHCl_3$ with methanol.

A yield of 6.5 mg. of large well formed crystals was obtained. Under the microscope, crushed crystals began to melt at 216.5°, new crystals forming in the melt as the temperature was increased slowly and all evidence of crystallinity disappearing at 218°. A mixed melting point of equal parts of three times crystallized mesoporphyrin dimethyl ester (prepared from heme of hemoglobin according to the method of Fischer and Pützer (3)) with the above meso ester crushed together on a cover-slip showed beginning of melting at 213.5°, the last crystals melting at 218.5°. These melting points may be considered as confirmation that the protoporphyrin isolated from the *Chlorella* cells is protoporphyrin isomer 9.

The absorption curve of the meso ester derived from the pigment of W_5 brown cells was compared with that of the meso ester prepared by the method of Fischer and Pützer (3) from blood heme. Within experimental error, the points on the mesoporphyrin curve are seen to be identical.

For comparison, the absorption curve of protoporphyrin dimethyl ester is also shown in Fig. 3. Because of the sensitivity of this compound to decomposition, it was recrystallized and then further purified by chromatographic adsorption in dim light on a column of 10 parts of powdered sucrose and 1 part of talcum. The column was first wetted with a 1:1 solution of petroleum ether (b.p. 70°) and benzene. The ester was dissolved in $CHCl_3$ and adsorbed on the column, which was then developed and eluted with a solution of $CHCl_3$ and benzene, the proportion of $CHCl_3$ in the solution being gradually increased. A pale greenish residue remained on the column. Addition of petroleum ether to the eluate resulted in beautiful plates arranged in rosettes which were dried *in vacuo* and used for the spectroscopic measurements on the same day.

Micromethod for Quantitative Conversion of Protoporphyrin to Mesoporphyrin Dimethyl Ester—Protoporphyrin has been found to be converted to mesoporphyrin quantitatively in 98 to 100 per cent formic acid in the presence of colloidal palladium and hydrogen in a period of 5 to 10 minutes, at 50–60°. No side products appear to be formed (at higher temperatures or in more aqueous formic acid, side products are observed) in contrast to results with the reduction methods with hydriodic acid (4).

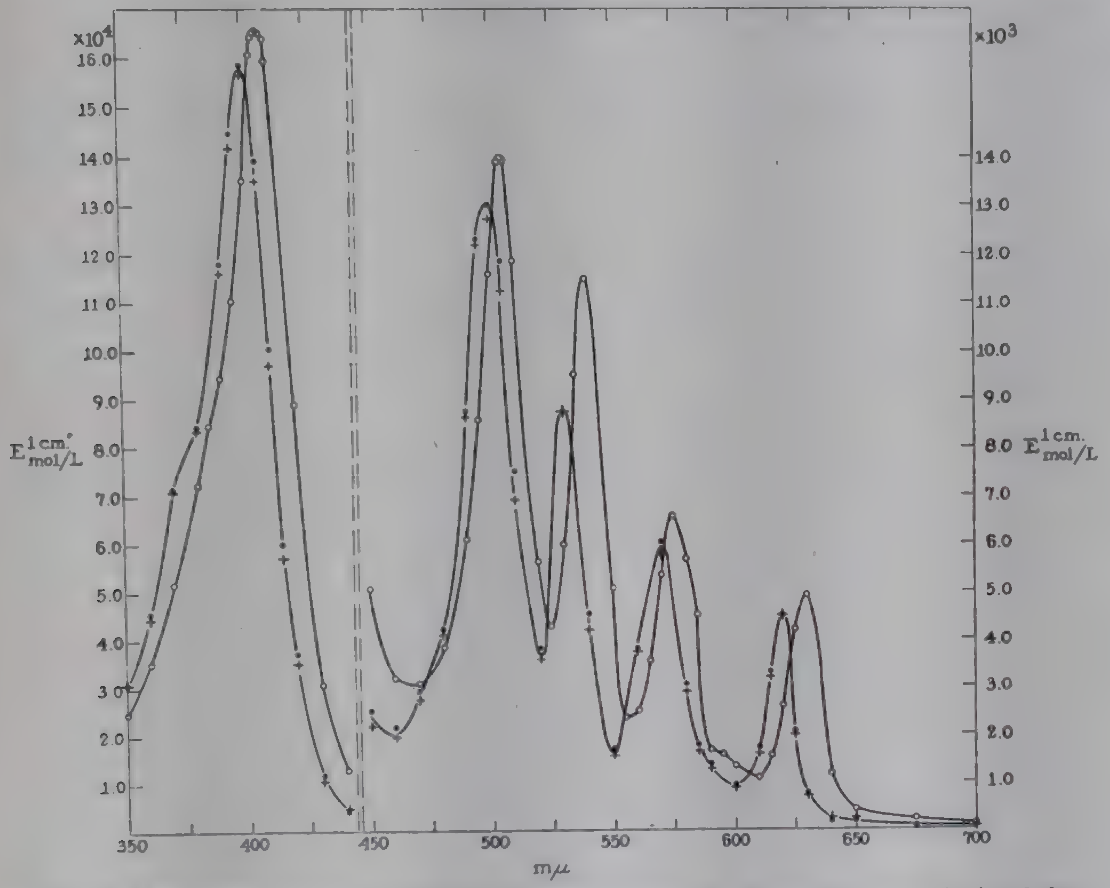


FIG. 3. Molar extinction in $CHCl_3$ of mesoporphyrin dimethyl ester derived from blood heme (●) and from the pigment of *Chlorella* W_5 brown mutant (+). For comparison of the positions of the bands and their maxima, the extinction of protoporphyrin dimethyl ester (○) in $CHCl_3$ is also given. The extinction is 10 times higher on the left side than on the right side. Molar extinction per cm. of light path for a given wave-length = $E^{1\text{ cm.}}_{\text{mole per liter}} = \log_{10} I_0/I$ (1/(cm. × mole per liter)).

For example, 11.4 mg. of protoporphyrin + 1.00 cc. of 99 per cent formic acid + 3.0 mg. of colloidal palladium (18 per cent Pd) were bubbled with hydrogen at 50–60° for 10 minutes in a small Claisen flask. The formic acid was then distilled off to complete dryness with the aid of a vacuum. Then 10 cc. of dry methanol-HCl (containing 5 per cent HCl by weight) were added, and the flask stoppered and left overnight at room temperature. The solution of mesoporphyrin dimethyl ester was transferred to a separatory funnel with 10 cc. of $CHCl_3$. The $CHCl_3$ solution was washed with water several times, then with 0.02 N NH_4OH, dried with anhydrous Na_2SO_4, filtered with a filter stick, and the $CHCl_3$ solution including the $CHCl_3$ washings of the Na_2SO_4 was evaporated down to a few cc., filtered, and further evaporated to dryness. The ester was then crystallized by dissolving it in 0.5 cc. of hot $CHCl_3$, adding 3 cc. of boiling methanol, letting the solution cool slowly in a beaker of hot water, and finally placing it in the ice box overnight. Large, well formed crystals resulted which were washed with dry methanol and were then dried in a vacuum desiccator at 70°. The yield was 10.63 mg. or 88.3 per cent of theory. (Further crystals were observed to form later in the mother liquor from the crystallization.) Crystals began to melt at 213.5° and complete melting was observed at 216°. The mixed melting point with ester derived from blood heme began to melt at 213.5°, complete melting being observed at 217°.

Melting Point of Mesoporphyrin 9 Dimethyl Ester—The identification of the isomeric porphyrins depends on the determination of the melting points of their methyl esters. The isomers are in general indistinguishable on the basis of absorption spectra. The melting points of twelve of the fifteen possible isomers are known (5). Two of these isomers have melting points in the neighborhood of those reported for isomer 9. Isomer 13 has a melting point of 217° and isomer 14 has a melting point of 205–209°. Fischer and coworkers have reported melting points for the mesoporphyrin 9 dimethyl ester as ranging from 206–216°. Taylor (6) has reported a value of 215.6°; "none of the samples melted with ideal sharpness." These variations and the unsharp melting point of the product derived from blood heme were at one time postulated by Fischer to represent a mixture of two isomers, but this idea was later rejected by him (7, 8). These facts suggest that the melting point criteria for the identification of the isomers have certain limitations

Using a melting point apparatus in conjunction with a polarizing microscope, we have observed the following phenomena. When the crystals of the meso ester were crushed very finely between two cover-slips and mounted in the hot stage, streaks of oriented material and small crystalline fragments could be seen under polarized light. As the temperature was raised and approached that of incipient melting, the streaks appeared to form tiny crystals oriented with their long directions parallel to the direction of the streaks. After melting had begun, one could see within the droplets numerous small crystals forming and melting. Only when the temperature had risen some 2–4° above the incipient melting point, did all birefringent material disappear. The incipient melting point was more easily seen at the edge of larger crystals.

For the calibration of the hot stage thermometer in this temperature range $AgNO_3$, m.p. 208°, was used. Although the $AgNO_3$ crystals could be seen to darken as the temperature was raised, the melting of all the crystals occurred within a rise of 0.5°. No organic material examined gave this sharp a melting point in this temperature range. The incipient melting points of a number of the meso ester preparations are listed in Table I.

TABLE I

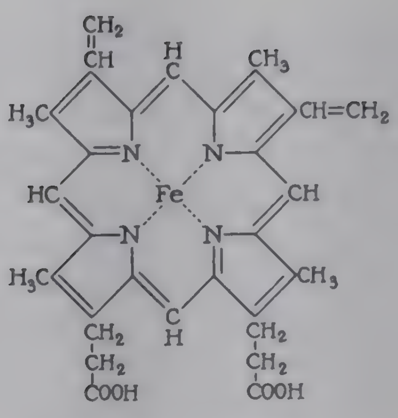
Incipient Melting Points of Mesoporphyrin 9 Dimethyl Esters Measured on Microscope Hot Stage Apparatus

Sample No.	Preparation	Incipient m.p.
		°C.
1	Ester prepared by method of Fischer and Pützer	210
2	Sample 1 recrystallized 3 times from $CHCl_3$-CH_3OH	212
3	Sample 1 chromatographed and recrystallized	216.5
4	Ester prepared from protoporphyrin 9 by Pd + H_2 + formic acid method	213.5
5	Ester prepared from W_5 brown extract by Pd + H_2 + formic acid method	216.5
6	Mixed m.p. of equal parts of Samples 5 and 2	213.5
7	" " " " " " protoporphyrin 9 ester (melting at 228°) with meso ester, Sample 2	214

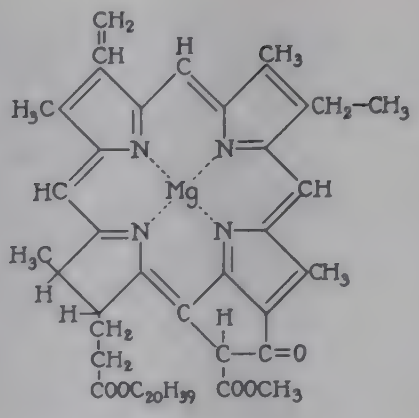
DISCUSSION

The lack of chlorophyll in the mutant *Chlorella* W_5 brown and the relatively high protoporphyrin content suggest the interpretation that a block has occurred in the synthetic chain, normally leading to chlorophyll, which permits the accumulation of the intermediate compound protoporphyrin.

Protoporphyrin has been detected in many animals and plants (9), existing either free in traces or generally in the form of the iron complex. Where it has been isolated from animal species in sufficient amounts, it has been found to be isomerically identical with protoporphyrin derived from the blood heme of mammals. In this paper, it has been shown that protoporphyrin derived from a plant cell, *Chlorella*, is also isomerically identical with the protoporphyrin of blood heme.



Fe protoporphyrin 9



Chlorophyll a

The similarity between the green pigment of the leaf, chlorophyll, and the red pigment of the blood, iron protoporphyrin, was hypothesized early by Hoppe-Seyler (10), Marchlewski (11), and others. It remained for Nenki, Piloty, Kuster, and Willstätter (12, 13) to recognize the basic plan of their ring structure and for Fischer, Conant, and their coworkers (14, 15) to determine the basic similarities of their side chain pattern.

The biochemical relationship between these two pigments is now clearly suggested by the work on *Chlorella*. Protoporphyrin appears to be the metabolic precursor of chlorophyll. A plausible series of oxidation-reduction and esterification steps may now be conceived for the biochemical synthesis of chlorophyll from protoporphyrin. It is hoped that the study of other mutants will demonstrate the order of this process.

We desire to acknowledge the invaluable technical assistance of Miss Ruth Kett and Mrs. Helen Brooks in the isolation and cultivation of the mutants. We also wish to express our thanks to Professor S. Trelease of Columbia University for his strain of *Chlorella vulgaris*, to Dr. S. H. Wollman of the x-ray department of Memorial Hospital for his kind cooperation, and to Dr. Leonor Michaelis for his constant stimulation and advice.

SUMMARY

A pigment isolated from an x-ray mutant of *Chlorella* has been identified as protoporphyrin by its absorption spectrum maxima, its HCl extraction number from ether solution, and its capacity to support the growth of an influenza organism. It was characterized as the 9 isomer by converting it to the protoporphyrin dimethyl ester and to the mesoporphyrin dimethyl ester, and by determining their melting points. The brown color of the cells was found to be due to the colloidal aggregation of protoporphyrin within them.

A quantitative micromethod is described for the conversion of protoporphyrin to mesoporphyrin by reduction of the vinyl groups with hydrogen and palladium in formic acid.

Protoporphyrin 9, the porphyrin of blood heme, is postulated to be a normal metabolic precursor in the synthesis of chlorophyll by the plant cell *Chlorella*.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF CITRIC ACID IN BLOOD AND PLASMA*

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As early as 1897 the oxidative bromination of citric acid to form a brominated acetone derivative, presumably principally pentabromoacetone, was adapted to the quantitative determination of citrate (1). Most subsequent procedures have been based on the same reaction. Among the more recently described procedures are those of Pucher (2) and of Goldberg and Bernheim (3). These methods, with minimum sensitivities of approximately 0.05 and 1.0 mg. of citric acid, respectively, are not sufficiently sensitive for many purposes; hence experiments were undertaken to develop a more sensitive procedure. In common with earlier procedures the method reported herein is based on the formation of a brominated acetone derivative. The sensitivity, however, has been lowered to 5 to 50 γ of citric acid by the use of a new procedure for the colorimetric microdetermination of bromide.

Since this work was completed, a method for citric acid determination with a sensitivity of 10 to 60 γ of citric acid has been published by Taussky and Shorr (4). This method, which also is based on the oxidative bromination of citric acid, appears satisfactory. The present procedure, however, is presented, as it differs considerably in the method used for the determination of the bromine present as brominated acetone derivatives.

EXPERIMENTAL

Reagents—

1. 1 N NaOCl in 0.1 N NaOH. 88 gm. of NaOH are dissolved in about 1500 ml. of water; the solution is cooled in an ice bath, and, with vigorous stirring, chlorine gas is bubbled through the solution until 71 gm. of chlorine have been added. The solution is then made up to 2 liters (5).

The concentration of NaOH should lie between 0.08 N and 0.12 N. It may be determined by adding 5.0 ml. of water and about 5 drops of 30 per cent hydrogen peroxide to a 1.0 ml. aliquot of the NaOCl solution, followed by titration with phenol red or another suitable indicator. The destruction of the NaOCl is complete when further addition of peroxide liberates no more oxygen bubbles.

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* The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

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2. 0.05 M sulfite and 0.40 M phosphate. 100 ml. of 0.50 M sodium sulfite (12.6 gm. of $Na_2SO_3 \cdot 7H_2O$ per 100 ml.), and 200 ml. of 2.0 M sodium dihydrogen phosphate (27.6 gm. of $NaH_2PO_4 \cdot H_2O$ per 100 ml.) per liter. Fresh solution must be prepared every 10 days and the curve standardized.

3. 6 M sodium formate solution. 408 gm. of HCOONa per liter.

4. 3 M H_3PO_4. 269 ml. of 85 per cent H_3PO_4 per liter.

5. Ammonium molybdate solution. 10.0 gm. of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per 100 ml.

6. 10 N sulfuric acid. 272 ml. of concentrated H_2SO_4 per liter.

7. 2 N hydrazine. 32.5 gm. of $N_2H_4 \cdot H_2SO_4$ are dissolved with the aid of 50 ml. of 5 N NaOH and made to 500 ml.

8. 1 M bromide-bromate solution. 90 ml. of 1 M KBr (119 gm. of KBr per liter) and 10 ml. of 1 M $NaBrO_3$ (15.1 gm. of $NaBrO_3$ per 100 ml.).

9. Petroleum ether, reagent grade, preferably with the boiling point above 60°.

10. Potassium iodide solution. 5.0 gm. of KI per 100 ml. Store in a cool, dark place. If a slight yellow color of free iodine appears, it should be discarded.

11. 0.1 M potassium permanganate. 15.9 gm. of $KMnO_4$ per liter.

12. 10.0 mM citric acid. 1.053 gm. of $C_6H_8O_7 \cdot H_2O$ and 50 ml. of 10 N H_2SO_4 per 500 ml.

13. 0.10 mM citric acid. 1.0 ml. of 10.0 mM citric acid and 1.0 ml. of 10 N H_2SO_4 made to 100 ml.

Standardization Procedure—For the preparation of a standard curve any satisfactory photoelectric colorimeter may be used. The curve is prepared as follows: In a 125 ml. separatory funnel are placed 5.0 ml. of 20 N H_2SO_4, 4.0 ml. of 8 per cent trichloroacetic acid, and 3.0 ml. of a series of standard citric acid solutions, containing from 5.0 to 50.0 γ of citric acid. The reagent blank is prepared in a similar manner except that water is substituted for the citric acid.

1 ml. of the 1 M bromide-bromate solution followed by 2.0 ml. of 0.50 M $KMnO_4$ is added at a very slow rate with continuous and vigorous agitation. The mixture is allowed to stand 10 minutes; then sufficient 2.0 M hydrazine solution (about 1.0 ml.) to decolorize the solution completely is added slowly, and the sides of the funnel are washed with 1 to 2 ml. of distilled water. 15 ml. of petroleum ether are added; the funnel is tightly stoppered with a clean rubber stopper and shaken vigorously for 5 minutes, preferably on an automatic shaker. After the extraction is completed, the pressure is released, the layers allowed to separate, and the aqueous phase is drawn off and discarded. The petroleum ether and the funnel

are washed four times with water, the stream of water from the wash bottle being directed first forcibly into the petroleum ether solution, then around the sides and top of the funnel. Water redistilled from an all-glass still is used for the washings. The extract is transferred to a clean separatory funnel, and the first funnel is rinsed with three small portions of petroleum ether. The extract is then washed twice more with water. Sufficient time is allowed after the last wash for complete separation of the aqueous layer which is then completely removed.

To the separatory funnel are added exactly 15 ml. of the sulfite-phosphate solution, as used by Goldberg and Bernheim (3). The funnel is tightly stoppered with a clean rubber stopper, and the mixture is vigorously shaken for 5 minutes. After separation of the layers, exactly a 10 ml. aliquot of the sulfite-phosphate solution is carefully transferred to a 25 ml. volumetric flask and the neck of the flask is rinsed with a few drops of water. The flask is heated in a boiling water bath for about 5 minutes, and exactly 2 ml. of the hypochlorite solution are added. The sides of the flask are rinsed carefully with two small portions (less than 1.0 ml.) of water, the contents are mixed, and the heating is continued for exactly 4 minutes from the time of the hypochlorite addition. Then 1.0 ml. of 6 M formate is added; the flask is removed from the bath and the contents carefully shaken. The flask is then rotated so that the hot formate solution comes in contact with the inner wall of the neck, and the solution is cooled to room temperature. 1 drop of ammonium molybdate solution is added to the flask and the sides of the flask are rinsed with 5.0 ml. of 3 M phosphoric acid. The contents are mixed, and 1.0 ml. of the potassium iodide solution is added. The contents are made to a 25 ml. volume, mixed, and transferred to a colorimeter tube. The developed color is determined 10 to 15 minutes after the iodide addition; in this investigation an Evelyn colorimeter was employed with the 400 mμ filter and with a blank prepared in the same manner as the sample.

Determination of Citric Acid in Blood—To 4.0 ml. of 10 per cent trichloroacetic acid in a centrifuge tube is added 1.0 ml. of fresh immediately drawn whole blood (oxalated or heparinized), or 1.0 ml. of plasma. The contents are vigorously shaken and allowed to stand 10 minutes. A 4.0 ml. aliquot of the clear centrifuged supernatant, representing 0.8 ml. of whole blood or plasma, is transferred to a 125 ml. separatory funnel. To this are added 5.0 ml. of 20 N sulfuric acid and 3.0 ml. of water. The solution is cooled to room temperature and the citric acid is determined as described above for standard solutions.

Results

In Fig. 1 is shown the standard curve obtained with pure citric acid solutions. A straight line is obtained by the plot of the L value ($2 - \log G$)

against the concentration. Duplicates agree within ±3 per cent. In Fig. 2 is shown the standard curve obtained with bromide solutions. This curve shows that the plot of optical density against concentration does not give a straight line with the instrument used. The straight line obtained with citric acid is fortuitous and probably depends on the known higher yield of the bromoacetone derivative with higher citric acid concentrations.

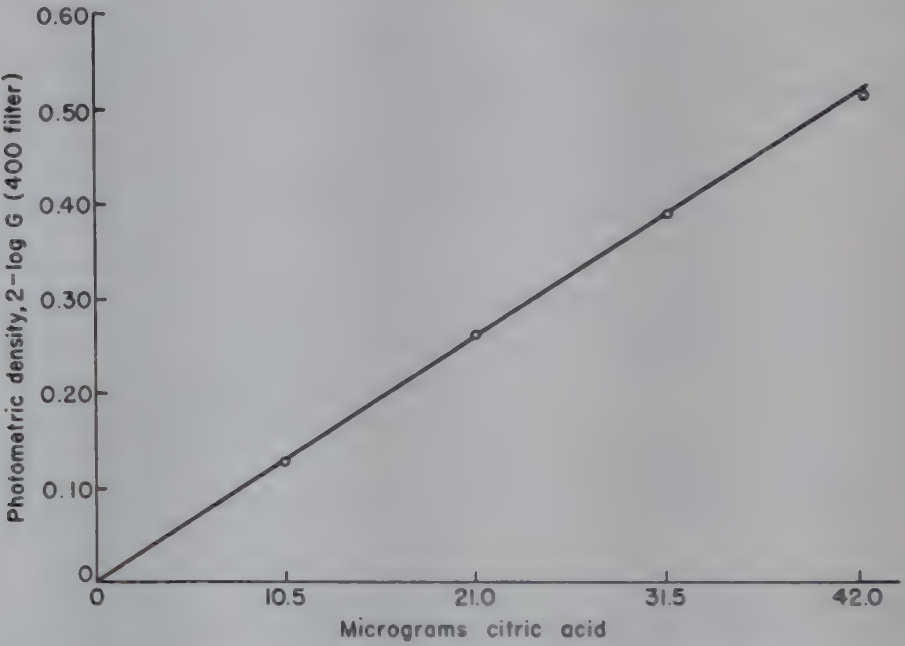

FIG. 1. Standard curve for pure citric acid solutions

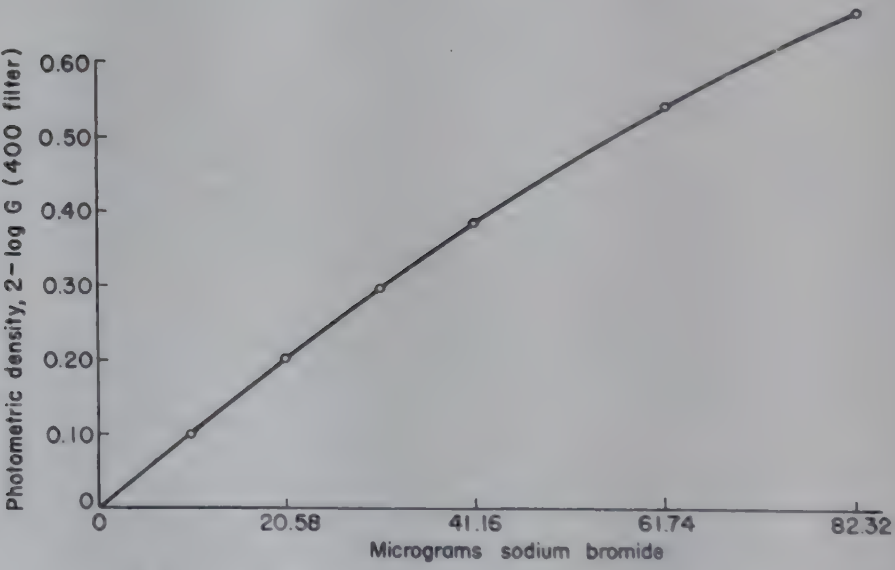

FIG. 2. Standard curve for bromide solutions

The values for citric acid in normal human blood are shown in Table I. Twenty-three normal and fasting whole blood samples were analyzed and the results range from 1.33 to 2.28 mg. of citric acid per 100 ml. of whole blood. Values are also presented for normal citric acid content of plasma, ranging from 1.60 to 2.66 mg. per 100 ml. of plasma.[1]

The partition of citric acid between plasma and cells, calculated on the basis of the hematocrit, shows a relative constancy, ranging from 64 to 82 per cent in plasma, with a mean of 76 per cent, and from 36 to 18 per cent in the cells (Table I).

The recovery of citric acid added to blood ranges from 97 to 102 per cent (Table II).

DISCUSSION

Interfering Substances—Low results are obtained in the presence of large amounts of glucose. Excessive amounts will be detected by an increased destruction of permanganate. The effect of large amounts of glucose (*i.e.*, 600 mg. per cent or more) can be readily eliminated by the modification of Goldberg and Bernheim (3). With normal blood glucose levels no special precautions are necessary.

Acetone bodies have been noted to interfere in similar methods by causing high results. If samples are known to contain acetone or acetoacetic acid (7), a 4.0 ml. trichloroacetic acid aliquot is boiled with 10.0 ml. of 10 N sulfuric acid until the volume is 10.0 ml. or less. The resultant volume is adjusted to 10 ml. with water and is quantitatively transferred to a separatory funnel with the aid of two 1.0 ml. water rinses. A preliminary bromination is carried out to eliminate ether-soluble brominated derivatives by the addition of 1 ml. of a bromide-bromate solution (50 ml. of a 1 M potassium bromide and 10.0 ml. of 1 M sodium bromate). The sample is allowed to stand for 30 minutes and then extracted with three 10.0 ml. aliquots of carbon tetrachloride. The carbon tetrachloride layer is drawn off and discarded, thus eliminating free bromine and ether-soluble brominated compounds. The citric acid determination is then carried out in the usual manner.

Formation of Bromoacetone Derivative from Citric Acid—The oxidation and bromination are made essentially as previously described (2, 3). The step involving addition of potassium permanganate is the most likely source of error, and slow addition of the permanganate is essential. The amount of bromine recovered as the bromoacetone derivative can be readily modified by variations in these conditions.

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[1] Natelson *et al.* (6) reported a method for the determination of citric acid after this manuscript was submitted for publication. The level of citrate in the red blood cells indicated by their results is somewhat lower than that reported herein.

TABLE I

Citric Acid Values in Normal Blood and Plasma

Subject	Cell volume	Citric acid			Per cent distribution, calculated	
		Whole blood	Plasma	Cells (calculated)	Plasma	Cells
	per cent	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*		
F. G.		2.25				
R. W.		2.28				
G. W.		2.05				
F. S.		1.81				
R. L.		2.26				
R. S.		2.11				
G. P.		1.94				
G. W.		1.96				
P. M		1.70				
G. W.		2.13				
P. M.		2.13				
G. P.		1.75				
M. H.	43	1.63	2.25	0.81	79	21
A. I.	44	1.61	2.29	0.75	79	21
R. F.	37	1.33	1.73	0.65	82	18
A. B.	41	1.70	2.19	1.00	76	24
R. F.	41	1.88	2.49	1.00	78	22
W. G.	44	1.98	2.66	1.11	75	25
M. T.	35	2.15	2.58	1.34	78	22
R. F.	38	1.38	1.63	0.97	73	27
L. G.	44	1.40	1.60	1.14	64	36
L. W.	42	1.75	2.33	0.95	77	23
R. S.	47	1.41	2.00	0.74	75	25

TABLE II

Recovery of Citric Acid Added to Human Blood

Subject	Citric acid present in 1.0 ml. whole blood*	Total citric acid		Per cent recovery
		Theoretical	Analytical	
	mg.	*mg.*	*mg.*	
G. W.	0.0164	0.0374	0.0370	98.1
R. W.	0.0182	0.0392	0.0386	97.1
F. S.	0.0145	0.0355	0.0349	97.1
R. L.	0.0181	0.0391	0.0394	101.4
G. W.	0.0157	0.0367	0.0371	101.9

* 0.0210 mg. of citric acid standard added in each case.

Adequacy of Petroleum Ether Extraction—Repeated extractions of the reaction medium with petroleum ether are unnecessary. A 2 minute period of vigorous shaking was found to extract completely all the bromoacetone derivative present. 5 minutes of shaking were used to insure complete extraction.

Washing of Petroleum Ether Solution and Funnel—The reaction mixture for the formation of the bromoacetone derivative contains high concentrations of bromide. Since the final color test is a measure of the small amount of bromine present as the bromoacetone derivative, very careful washing of the separatory funnels and of the extracts is necessary. Bromide is very sparingly soluble in petroleum ether, and contamination is most likely to come from inadequate washing of the funnel. If the washing is satisfactory, a blank carried through the entire procedure will show no more color than a blank for the hypochlorite oxidation only.

Adequacy of Sulfite Extraction—Although a maximum amount of bromide from the pentabromoacetone is transferred to the sulfite solution by 5 minutes extraction, some of the bromine in the aqueous phase may still be in organic combination. This is indicated, since the sulfite extract gave slightly higher results if given a preliminary heating before the hypochlorite addition. Hence samples were routinely placed in the boiling water bath about 5 minutes before the hypochlorite addition.

Oxidation of Bromide to Bromate—Comparative tests showed that all the bromide is oxidized to bromate under the given experimental conditions within 2 minutes at the temperature of the boiling water bath. The heating period was extended to 4 minutes to insure complete oxidation.

Equivalent quantities of bromide added before heating with hypochlorite or of bromate added after heating with hypochlorite yielded exactly the same amount of color. The amount of hypochlorite added is sufficient to oxidize all the sulfite and to leave an excess for oxidation of the bromide. The oxidation by hypochlorite is interfered with by glycerol and possibly by fairly large quantities of other similar substances. This interference was noted when glycerol was used to seal the stoppers of the separatory funnels during extractions.

Reduction of Hypochlorite with Formate—The decomposition of the excess hypochlorite by the formate takes place very rapidly at 90–100°, but much more slowly at room temperature. Thus it is necessary to add the formate before cooling the samples.

Conditions for Color Development—It was desired to find conditions under which the iodine liberated by reaction with the bromate could be used for the colorimetric measurement. The colorimetric measurement is preferable to the titrimetric technique, since it is faster, more sensitive, and precise for the small concentrations of iodine involved. Under conditions customarily used in titrimetric procedures, continued liberation of iodine from the excess of iodide proceeds at a rate far too rapid for satisfactory colorimetric measurement. It was found that at a higher pH and with the addition of molybdate as a catalyst the liberation of iodine reached a plateau within 10 minutes, after which subsequent color increase was very slow. Thus a precise colorimetric estimation is possible. Close regulation of the pH is necessary. In the above procedure regulation of the pH is accomplished by the use of sodium dihydrogen phosphate in the sulfite extraction medium together with the phosphoric acid just before the color development to form a buffer of an appropriate pH.

Both sulfite and hypochlorite will interfere in the color development, sulfite by reducing the liberated iodine, and hypochlorite by liberating iodine from the excess iodide. Thus it is important that all the sulfite be oxidized by the hypochlorite and that the hypochlorite be completely reduced by the formate.

SUMMARY

1. A colorimetric method has been described for the determination of 5 to 50 γ of citric acid in whole blood and plasma.

2. The method is a modification of previous methods based on the conversion of citric acid into a bromoacetone derivative, together with a new procedure for the microcolorimetric determination of the amount of bromine present as the bromoacetone derivative.

We gratefully acknowledge the valuable assistance of Mr. S. E. Lenz. The work was initiated at the suggestion of Dr. Eugene Lozner. Also it is a pleasure to acknowledge the valuable contribution of Miss Sonia Lemish to early phases of the work.

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INFLUENCE OF INSULIN PREPARATIONS ON GLYCOGENOLYSIS IN LIVER SLICES*

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Shipley and Hümel (1) have shown that addition of insulin to rat liver slices with high glycogen content incubated in serum causes an increase in sugar output when compared with liver slices incubated without insulin. Although this observation has been confirmed, it has been found that the glycogenolytic action is not related to the ability of insulin preparations to produce hypoglycemia when injected into the intact animal. This follows from the fact that the hypoglycemic action of insulin preparations can be destroyed by treatment with cysteine or with alkali without destroying the glycogenolytic effect. Furthermore, a Danish insulin preparation, which caused hypoglycemia when injected into animals, had no glycogenolytic action on liver slices.

The experiments reported in this paper show that liver slices incubated *in vitro* represent a sensitive and reproducible test system for a glycogenolytic factor which is present in highly purified amorphous as well as in crystalline zinc insulin preparations.

Methods

Well fed rats or rabbits were bled under ether or pentobarbital anesthesia and part of the liver was rapidly excised and placed in cold isotonic solutions of sodium chloride or potassium chloride. After several minutes of chilling,[1] a section of the liver was placed and held firmly between cold ground glass surfaces. Slices were prepared from such sections, and the slice thickness was estimated by watching the razor blade as it sliced through the upper portion of the liver section in contact with the ground glass surface. The slices in any one experiment were from the same section of the liver and were matched as closely as possible. The individual slice, approximately 1 cm. in length and width, was weighed on a torsion balance. Most slices weighed in the neighborhood of 70 mg.; thinner slices of 40 to 50 mg. and thicker slices of approximately 130 mg. weight were also used

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[1] The liver may be allowed to stand in *cold* saline for some time before slicing, but should not be kept at room temperature longer than is necessary.

successfully. In most cases one slice was incubated in a Warburg vessel containing 1.6 cc. of fluid shaken at the rate of 110 oscillations per minute. Both aerobic and anaerobic experiments were carried out. Incubation temperature was 37° in all cases. In most experiments the final insulin concentration was approximately 0.03 mg. per cc.[2]

Several buffers were used, but usually an isotonic chloride-phosphate solution with sodium or potassium cations was employed. The glucose output from the slices was measured by determining glucose in the medium after deproteinizing an aliquot with barium hydroxide and zinc sulfate. The Nelson method (2) was used for the sugar determinations. Glycogen was determined by the method of Good, Kramer, and Somogyi (3), the glucose in the acid hydrolysate being determined by the Nelson method. Phosphate was determined by the method of Fiske and Subbarow (4). Protein was determined by the biuret method of Weichselbaum (5).

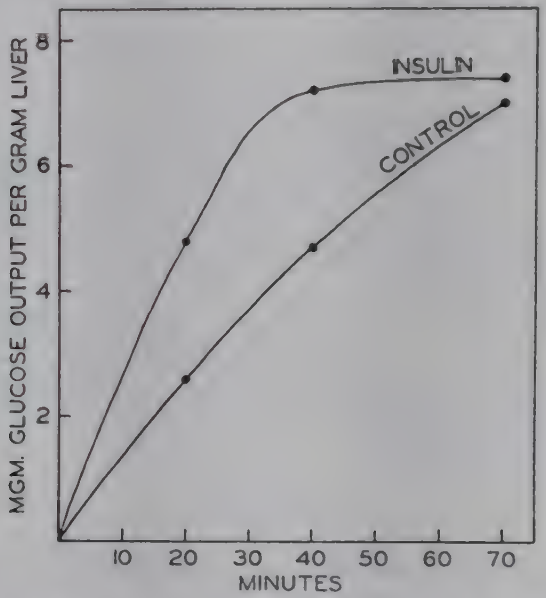

FIG. 1. Time curve of glycogenolytic effect of insulin in slices of rat liver. Slices were incubated in test-tubes containing 11.0 cc. of $NaCl-K_2HPO_4$ buffer, pH 7.5. O_2 was constantly passed through the buffer and aliquots of media were taken for analysis at times indicated. The insulin concentration was 0.055 mg. per cc.

Glucose Output from Liver Slices of Different Species—Rat liver slices incubated aerobically were used in preliminary experiments. With these an increased sugar output can be seen regularly in the presence of insulin if a time curve is made. An example is shown in Fig. 1; it emphasizes the importance of choosing the optimal time for sampling, because with time the sugar output of the control slices may reach that of the slices incubated with insulin. In twenty-two comparisons with slices from nine rats, the average increase in the glucose output with insulin was 31 per cent. Two of these comparisons showed an equal glucose output with and without insulin; none showed decreased output with insulin. Shipley and Hümel observed a doubling of the sugar output after 2 hours of incubation with insulin but it should be noted that they incubated the slices in serum and not in phosphate buffer.

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[2] The insulin used in the present investigation was of three types: Insulin A, crystalline zinc insulin supplied by Eli Lilly and Company; insulin B, a highly purified amorphous preparation (20 units per mg.) supplied by Eli Lilly and Company; and insulin C, crystalline zinc insulin prepared by Novo Terapeutisk Laboratorium, Denmark. For brevity's sake, unless otherwise specified, the term "insulin" refers to insulin A or B, both of which contain the glycogenolytic factor.

The glycogenolytic effect of insulin on rabbit liver slices is more marked than on rat liver slices and can be demonstrated after short as well as after long incubation periods. In fourteen aerobic and twenty-four anaerobic experiments, insulin increased the glucose output in each case, on an average of 135 per cent. The slices were prepared from the livers of twenty-three rabbits. For some of the anaerobic experiments commercial nitrogen was used without further purification. Since there was still some O_2 consumption present, nitrogen purified by passage over heated copper was used in later experiments. Table I summarizes the experiments on the effect of insulin on the glucose output from rabbit and rat liver slices.

TABLE I

Effect of Insulin on Glucose Output from Rabbit and Rat Liver Slices

The results are expressed in per cent increase in glucose output on comparing control slices with slices incubated with insulin. The amount of insulin added was 0.006 to 0.08 mg. per cc., usually 0.03 mg. per cc.

Species	No. of experiments		Increase with insulin	
	Aerobic	Anaerobic	Range	Average
			per cent	*per cent*
Rabbit	4	2	20– 50	33
	5	17	50–150	105
	5	5	150–400	262
Rat	17		0– 50	19
	5		50–100	72

One aerobic experiment with cat liver slices gave an increased output of glucose in the insulin sample amounting to over 200 per cent and in one aerobic experiment with pigeon liver slices the increase was 170 per cent. Subsequent experiments were carried out with rabbit liver slices.

Relation to Glycogen Breakdown—Glycogen analysis showed that the increased glucose production with insulin was accompanied by an increased rate of glycogen breakdown in the slices. In Table II it may be seen that approximately 60 per cent of the glycogen which disappeared was found as glucose in the fluid in which the slices were shaken. The loss of carbohydrate by oxidation and lactic acid formation was not taken into account.

Buffers—The glycogenolytic effect of insulin was observed in all buffers employed. These included (1) isotonic chloride-phosphate buffers with cation all sodium, all potassium, or a mixture of both, (2) chloride-phosphate buffers plus bicarbonate, (3) mammalian Ringer's solution plus phosphate, and (4) Krebs' phosphate buffer (with no calcium added). The pH range was from 7.2 to 7.6. In two experiments a glycogenolytic effect of insulin was obtained at pH 6.4, although it was somewhat less than the effect seen in duplicate experiments at pH 7.4.

TABLE II

Relation of Glycogen Disappearance to Glucose Output

Single slices from the same section of a rabbit liver were incubated aerobically for 60 or 110 minutes. Insulin concentration was 0.05 mg. per cc. After incubation, an aliquot of the medium was taken for glucose analysis; the remainder, including the slice, was heated with 30 per cent NaOH for glycogen analysis. In the case of glycogen analysis, the glucose output was calculated from the difference in glycogen content before and after incubation.

Additions	Time of incubation	Glucose output per gm. liver	Glucose output per gm. liver minus control	Method of analysis
	min.	*mg.*	*mg.*	
None	60	9.1		Glucose
Insulin	60	12.1	3.0	"
None	60	15.7		Glycogen
Insulin	60	21.4	5.7	"
None	110	18.3		Glucose
Insulin	110	24.1	5.8	"
None	110	28.8		Glycogen
Insulin	110	39.8	11.0	"

Effect of Cell Disruption—Homogenization of the liver caused disappearance of the glycogenolytic effect of insulin. Twenty animals were used in homogenization experiments, including cats, rabbits, rats, and one pigeon. Variation of media, degree of homogenization, and centrifugation were tried, but with one exception resulted in negative experiments. Freezing the tissue, either before or after slicing, had relatively little effect on the glucose output without insulin, but caused a disappearance of the accelerating effect of insulin on glycogenolysis. An example is shown in Table III.

Anaerobiosis—The glycogenolytic effect of insulin on rabbit liver slices can be demonstrated under strictly anaerobic conditions (Table IV). In

Experiment 1, where the glycogenolytic effect of insulin is compared under anaerobic and aerobic conditions, it may be seen that the effect is only half as great in the former as in the latter case. In Experiment 2 it is shown that the glycogenolytic effect of insulin is less after 5 minutes of preliminary anaerobic incubation of the liver slices and disappears almost completely after 20 minutes of preliminary anaerobic incubation.

TABLE III

Effect of Insulin on Liver Slices Previously Frozen

Liver slices were frozen at −20° and a few minutes later were thawed and compared with unfrozen slices from the same rabbit liver. The time of incubation was 45 minutes.

Additions	Experimental conditions	Glucose output per gm. liver	Glucose output per gm. liver minus control
		mg.	*mg.*
None		1.4	
Insulin		5.4	4.0
None	Previously frozen	2.4	
Insulin	“ “	2.9	0.5

TABLE IV

Effect of Insulin under Anaerobic Conditions

In Experiment I rabbit liver slices were incubated in air, or in nitrogen purified by passage over heated copper. Insulin was added from the side arm at the start of the incubation period. In Experiment 2 insulin was added from the side arm at 0 time, after 5 and 20 minutes. The total period of incubation was 40 minutes in both experiments.

Experiment No.	Additions	Gas phase	Glucose output per gm. liver	Glucose output per gm. liver minus control
			mg.	*mg.*
1	None	Air	10.2	
	Insulin	“	16.4	6.2
	None	Nitrogen	8.4	
	Insulin	“	11.5	3.1
2	None	“	5.1	
	Insulin at 0 incubation time	“	11.3	6.2
	“ “ 5 min. incubation time	“	9.9	4.8
	“ “ 20 “ “ “	“	5.7	0.6

As shown in Table IV, Experiment 1, less glucose is formed anaerobically than aerobically, even when no insulin is added. This confirms a similar observation made by Jeener (6), who made a detailed study of this phenomenon and concluded that there was a greater loss of adenylic acid in liver slices under anaerobic than under aerobic conditions and that this would influence the rate of glycogenolysis, since adenylic acid activates phosphorylase. Accordingly, the effect of addition of adenylic acid to liver slices has been tried, but a clear cut effect on the rate of glycogenolysis or on the magnitude of the glycogenolytic effect of insulin could not be demonstrated during 1 hour of incubation.

Point of Action—Table V presents three experiments in which an attempt was made to localize the action of insulin on glycogenolysis. Experiment 1 shows that addition of phosphate is necessary for the full glycogenolytic effect of insulin, which points to an action on the phosphorylase system. In Experiment 2, slices were incubated in a saline phosphate buffer without or with the addition of glucose-1-phosphate. The slices were able to dephosphorylate glucose-1-phosphate in addition to the ester supplied from glycogen by phosphorylase activity.[3] This indicates that phosphorylase, rather than phosphoglucomutase and phosphatase, is limiting the over-all rate of glucose formation from glycogen. The same

TABLE V

Effect of Inorganic Phosphate and Glucose-1-Phosphate on Glucose Output in Rabbit Liver Slices

In Experiment 1 the slices were incubated in bicarbonate buffer, in Experiments 2 and 3 in saline phosphate buffer.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control	Time of incubation
		mg.	*mg.*	*min.*
1	None	2.1		40
	Insulin, 0.03 mg.	3.3	1.2	40
	None	2.4		55
	Insulin, 0.03 mg.	4.4	2.0	55
	Phosphate, 0.06 cc. 0.1 M	2.2		50
	“ 0.06 “ 0.1 “ + insulin, 0.03 mg.	7.6	5.4	50
2	None	1.3		45
	Insulin, 0.06 mg.	4.7	3.4	45
	Glucose-1-PO_4, 3.0 mg.	4.4		50
	“ 3.0 “ + insulin, 0.06 mg.	8.1	3.7	50
3	None	4.7		60
	Insulin, 0.05 mg.	5.2	0.5	60
	Glucose-1-phosphate, 40 mg.	30.4		60
	“ 40 “ + insulin, 0.05 mg.	31.3	0.9	60

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[3] It is assumed that the reactions involved in glucose formation are glycogen + PO_4 → glucose-1-PO_4 → glucose-6-PO_4 → glucose + PO_4.

result was obtained in Experiment 3, in which a high concentration of glucose-1-phosphate was added to a liver slice from a fasted rabbit.[4] In homogenates, as in slices, the phosphorylase activity was again found to be the limiting step for the rate of glucose formation from glycogen. Insulin, as shown in Table V, had no effect on dephosphorylation of glucose-1-phosphate.

The glycogenolytic effect of insulin appears to be on the phosphorylase system and is dependent on an intact cell structure; this makes it difficult to investigate the mechanism of this insulin effect.

TABLE VI

Absence of Glycogenolytic Effect of Other Proteins on Rabbit Liver Slices

The time of incubation was 45 minutes.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1	None	2.4	
	Insulin, 0.03	5.0	2.6
	Dialyzed rabbit serum, 0.09	3.0	0.6
	" " " 0.09 + insulin, 0.03	6.2	3.8
2	None	1.9	
	Insulin, 0.06	9.0	7.1
	Casein, 0.18	2.0	0.1
3	None	2.4	
	Insulin, 0.03	5.7	3.3
	Triose dehydrogenase, 0.03	2.3	−0.1
	Zein (suspension), 0.06	2.4	0.0
	Commercial hydrolysate wheat gluten, 0.06	2.7	0.3
4	None	2.6	
	Insulin treated with alkali, 0.05	5.8	3.2
	Ribonuclease, 0.04	2.8	0.2
	" treated with alkali, 0.06	2.3	−0.3
	Egg albumen " " " 0.06	2.1	−0.5

Specificity—A number of proteins and other substances were added to the slices and their effect on the glucose output was measured. All additions failed to reproduce the effect of insulin (Table VI). With dialyzed rabbit serum, a slight increase in the glucose output was noted. Insulin in the presence of serum appeared to have a larger glycogenolytic effect than in its absence. Other substances not included in Table VI which were tested with negative results were cysteine, cystine, adenylic acid, coenzyme I, riboflavin, nicotinamide, and folic acid. It would appear that the glycogenolytic effect of insulin preparations is rather specific.

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[4] When liver slices are incubated with glucose-1-phosphate plus fluoride, a rapid synthesis of glycogen takes place within the slice (7). This shows that glucose-1-phosphate is able to diffuse into the liver cells.

The next question was whether or not the glycogenolytic effect was related to the hypoglycemic action of insulin. In Table VI, Experiment 4, it is shown that insulin inactivated by treatment with alkali still produced a glycogenolytic effect, while other proteins treated in the same manner had no effect on hepatic glycogenolysis. Further experiments are given in the next section.

TABLE VII

Effect of Varying Amounts of Insulin before and after Treatment with Cysteine or Alkali

Rabbit liver slices were incubated for 40 minutes.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1	None	2.3	
	Insulin, 0.0006	2.4	0.1
	" 0.03	5.1	2.8
	" 0.06	5.6	3.3
	" treated with cysteine, 0.03	5.3	3.0
2	None	2.4	
	Insulin, 0.006	4.6	2.2
	" 0.03	5.0	2.6
	" treated with cysteine, 0.03	5.6	3.2
3	None	2.8	
	Insulin, 0.003	4.5	1.7
	" 0.03	6.0	3.2
	" treated with alkali, 0.003	4.5	1.7
	" " " " 0.03	7.1	4.3

Glycogenolytic Effect of Inactivated Insulin—Insulin was treated with cysteine or alkali to the stage where no hypoglycemic action could be demonstrated in the intact animal. Cysteine inactivation was carried out in Thunberg tubes for 2 to 20 hours with a cysteine to insulin ratio of 40:1 by weight. Excess cysteine was present at the end of the incubation time. Alkali-inactivated insulin usually was prepared by incubating insulin with 0.08 N KOH for 3 hours at 36°. Four rabbits (two fasted and two fed) were injected intravenously with 1.6 to 2.0 mg. of the alkali-treated insulin. Blood sugars were determined for 3 hours after injection. In no case was any hypoglycemia observed. 30 minutes after injection the average blood sugar level was 20 per cent above the level before injection.

Table VII shows that the glycogenolytic effect of varying amounts of insulin before and after incubation with cysteine or alkali remained practically the same.

It has been shown previously that alkali-inactivated insulin loses its effectiveness in the hexokinase test system (8). The limiting reaction for the uptake of glucose by the isolated rat diaphragm (Gemmill (9)) is probably the hexokinase reaction. In twenty-two experiments on rat diaphragms a stimulation of glucose uptake could not be demonstrated with alkali-inactivated insulin, while stimulation was regularly observed with native insulin.

Properties of Insulin Inactivated by Alkali—When insulin solutions after incubation in 0.08 N KOH were adjusted to pH 5.8 HCl, hydrogen sulfide was released and some protein was precipitated. The precipitate was removed and trichloroacetic acid was added to the clear supernatant fluid to give a final concentration of the acid of 5 per cent. A second precipitate formed which was removed and was found to be active in the glycogenolytic test. The filtrate was shaken with ether to remove the trichloroacetic acid. On the basis of quantitative biuret determinations it contained about 15 per cent of the alkali-treated insulin protein and per unit of protein was about one-half as active in the glycogenolytic test as the original insulin. The trichloroacetic acid filtrate of native insulin did not contain material giving the biuret reaction and did not accelerate glucose output in liver slices (Table VIII).

When alkali-incubated insulin was neutralized and dialyzed in a cellophane sac for 24 hours at 5°, 2 to 3 per cent of the protein was recovered in the dialysate on the basis of biuret determinations. The dialysate was about as active per unit of biuret reading as the undialyzed material. The dialysate of native insulin did not contain glycogenolytically active material (Table VIII). The change in the properties of the glycogenolytic factor after treatment with alkali (incomplete precipitation with trichloroacetic acid, partial diffusibility through a cellophane membrane) suggests that it can be degraded by alkali to some extent without losing its activity. The glycogenolytic activity of insulin was lost after more rigorous treatment with alkali (40 minutes at 100° in 0.08 N KOH). The fact that the glycogenolytic factor in alkali-treated insulin lost its activity on incubation with trypsin (Table IX) suggests that it is a protein or polypeptide.

Experiments with Insulin C—It has been reported by deDuve, Hers, and Bouckaert (10) and by Olsen and Klein (11) that a Danish insulin in contrast to certain other commercial insulin preparations did not produce an initial hyperglycemia when injected intravenously into the intact animal.

A sample of this insulin, kindly supplied by Dr. Olsen, was tested for its glycogenolytic effect on liver slices. Since this preparation contained considerable amounts of preservative, dialyzed preparations were used in all but preliminary experiments. Dialyzed preparations of insulin A[2] were used as controls. The dialyzed sample of the Danish insulin, insulin C,[2] while possessing hypoglycemic activity in the intact animal, did not accelerate glycogenolysis in liver slices. Examples are given in Experiments 1 and 2, Table X. Experiment 3 shows that insulin C does not prevent the glycogenolytic effect of added insulin A.

TABLE VIII

Properties of Products Formed by Treatment of Insulin with Alkali

Rabbit liver slices were incubated for 45 minutes. In Experiment 1, insulin, or insulin pretreated with alkali, was precipitated with trichloroacetic acid and the filtrate, after removal of the trichloroacetic acid with ether, was added. In Experiment 2, a dialysate of alkali-treated or native insulin was added.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1	None	1.7	
	Alkali-treated insulin-trichloroacetic acid filtrate, 0.014	3.1	1.4
	Alkali-treated insulin-trichloroacetic acid filtrate, 0.140	4.2	2.5
	Native insulin-trichloroacetic acid filtrate (0.06 cc.)*	2.4	0.7
	Insulin, 0.006	3.4	1.7
	" 0.06	4.7	3.0
2	None	3.5	
	Alkali-treated insulin dialysate, 0.006	6.0	2.5
	Native insulin dialysate (0.12 cc.)*	3.8	0.3
	Alkali-treated insulin, 0.03	5.8	2.3
	" " 0.3	6.1	2.6

* The volume in cc. was equivalent to that added to the liver slices in the experiment represented in the preceding line.

When insulin C was incubated with 0.08 N KOH at 36° for 3 hours and for 16 hours, or with 0.1 N HCl for 16 hours, it did not acquire glycogenolytic properties (Table XI).

It has been possible to demonstrate where the separation of the glycogenolytic component occurs in the process of the purification of insulin. Four insulin preparations (in solid form) were kindly supplied by Dr. Hallas-Moeller of the Novo laboratories. Experiments with these preparations are shown in Table XII. It may be seen that the glycogenolytic activity is not lost when the amorphous insulin I is recrystallized by a modification of the Scott and Fisher method (12), but is completely lost when recrystallized by the method used by the makers of insulin C.

In confirmation of these results it has been found that the glycogenolytic activity of insulin A is not readily lost by isoelectric precipitations, or recrystallization from phosphate buffers. In one experiment with the Abel method (13) of purification, considerable separation was achieved. The Abel method was followed exactly until a few crystals had formed. These were removed and water was added dropwise to the heavily buffered solution until a slight haze appeared. A partly amorphous, partly crystalline

TABLE IX

Effect of Crystalline Trypsin on Products of Alkali-Treated Insulin

Rabbit liver slices were incubated for 45 minutes.

Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
mg.	*mg.*	*mg.*
None	1.5	
Alkali-treated insulin, 0.025	3.4	1.9
" " 0.025, after incubation with trypsin, 0.016	1.6	0.1
Alkali-treated insulin, 0.025, plus trypsin* 0.016	3.8	2.3

* In this case trypsin was added at the start of the incubation period of the liver slices.

TABLE X

Absence of Glycogenolytic Effect in Insulin C

Rabbit liver slices were incubated for 45 minutes.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1	None	9.1	
	Insulin C, 0.06	9.3	0.2
	" B, 0.05	15.6	6.5
2	None	9.0	
	Insulin C, 0.06	9.7	0.7
	" A, 0.006	15.4	6.4
3	None	7.4	
	Insulin C, 0.035 plus insulin A, 0.03	13.9	6.5
	" A, 0.03	13.0	5.6

TABLE XI

Insulin C after Incubation with Alkali and Acid

Rabbit liver slices were incubated for 40 minutes.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1	None	6.1	
	Insulin C, treated with 0.08 N KOH for 3 hrs. at 36°, 0.06	6.0	−0.1
	Insulin B, 0.05	11.4	5.3
2	None	6.7	
	Insulin C, treated with 0.08 N KOH for 16 hrs. at 36°, 0.045	6.6	−0.1
	Insulin C, treated with 0.1 N HCl for 16 hrs. at 36°, 0.045	6.4	−0.3
	Insulin B, 0.045	15.0	8.3

TABLE XII

Loss of Glycogenolytic Activity on Recrystallization of Insulin

Rabbit liver slices were incubated for 45 minutes.

Experiment No.	Additions per cc.	Glucose output per gm. liver	Glucose output per gm. liver minus control
	mg.	*mg.*	*mg.*
1*	None	5.7	
	I. Amorphous insulin from makers of insulin C, about 15 units per mg., 0.06	10.4	4.7
	II. Crystalline insulin (recrystallized) prepared from I by modified method of Scott and Fisher, 0.06	9.3	3.6
	III. Crystalline insulin, prepared from I by makers of insulin C, 0.06	8.3	2.6
	IV. Recrystallized from III by makers of insulin C, 0.06	5.2	−0.5
	Insulin B, 0.06	10.0	4.3
2	None	5.1	
	Insulin I, 0.06	13.2	8.1
	" II, 0.06	11.0	5.9
	" III, 0.06	9.0	3.9
	" IV, 0.06	5.2	0.1
	" B, 0.06	10.5	5.4

* All the results are the averages of two paired liver slices.

precipitate formed which had no glycogenolytic activity when tested in a concentration of 0.03 mg. of protein per cc. but had some activity when tested in a concentration of 0.13 mg. per cc.

DISCUSSION

The initial hyperglycemia which has been observed in intact animals after intravenous injection of various insulin preparations (14) and the glycogenolytic effect of insulin on isolated liver slices appear to be related phenomena. This follows from the fact that a Danish insulin[2] which fails to produce initial hyperglycemia in intact animals also fails to cause increased glycogenolysis in liver slices.

There are a number of questions raised by the presence of a potent glycogenolytic factor in highly purified insulin preparations which cannot be answered at the present time. One would like to know whether the factor, if originally present in the pancreas, has any physiological significance and whether previous experimental results, particularly those relating to the effect of insulin on liver glycogen in intact animals, were influenced by the presence of the glycogenolytic factor. Finally, the possible effect of the glycogenolytic factor after intravenous injections of large doses of insulin in man should not be overlooked.

The *in vitro* effects of insulin on the glucose uptake by the isolated diaphragm and on the hexokinase reaction were shown to be abolished by treatment of insulin with alkali. This would indicate that they are caused by insulin itself. It remains to be seen whether this is true for other *in vitro* effects of insulin, for example those on respiration and on gluconeogenesis.

SUMMARY

1. Highly purified amorphous as well as crystalline zinc insulin preparations contain a factor other than insulin which increases the rate of conversion of glycogen to glucose in isolated liver slices suspended in phosphate buffer. The average increase with rabbit liver slices was over 100 per cent, and 0.006 mg. of the above insulin preparations per cc. of medium gave a nearly maximal response. The glycogenolytic effect of "insulin" could not be reproduced by other proteins, by a protein hydrolysate, or by a number of other substances.

2. When the cell organization was disrupted by grinding or by freezing and thawing, or when the slices were kept anaerobically for 20 minutes at 37°, "insulin" failed to exert a glycogenolytic effect.

3. Phosphorylase activity was found to be the limiting step for the reaction, glycogen → glucose, in liver slices (and homogenates) which indicates that the glycogenolytic factor acts on the phosphorylase system.

4. Insulin treated with cysteine or alkali to a point when it no longer produced hypoglycemia in intact animals did not lose its glycogenolytic effect. In native insulin (but not in alkali-inactivated insulin) the glycogenolytic factor was completely precipitated by trichloroacetic acid and was not dialyzable; its destruction by trypsin suggests that it is a protein.

5. A Danish insulin preparation was found to be free of the glycogenolytic factor when 0.06 mg. per cc. was added. This was shown to be due to a special method of crystallization used by the manufacturers of this insulin. Isoelectric precipitation or recrystallization of crystalline zinc insulin did not lead to a separation from the glycogenolytic factor, while purification by the method of Abel *et al.* was partially successful.

6. It is concluded that the initial hyperglycemia which is observed in intact animals after intravenous injection of certain insulin preparations is caused by a glycogenolytic factor which acts directly on the liver.

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BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

VII. RELATIONSHIP OF PURINES AND THYMINE TO FOLIC ACID

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(Received for publication, September 26, 1947)

Snell and Mitchell (1) and Stokstad (2) have shown that thymine and purines are capable of promoting growth of *Streptococcus faecalis* R and *Lactobacillus casei* in the absence of an exogenous supply of folic acid. Stokes (3) was unable to detect folic acid in cells of such organisms after growth in a thymine- and purine-containing medium but did detect the presence of folic acid in cells after growth in a folic acid-containing medium. On the basis of these data and in view of the small concentration of folic acid necessary to replace the thymine requirement, Stokes suggested that folic acid functions as "a coenzyme for the enzyme system responsible for the synthesis or thymine (or more probably a thymine-like compound) which in turn is used by the bacteria to form nucleic acid."

Hitchings, Falco, and Sherwood (4) pointed out that "this hypothesis is not an adequate explanation of the action of thymine" in view of their results with analogues of thymine. The toxicity of some of the thymine analogues for *Lactobacillus casei* was prevented by increased concentrations of either folic acid or thymine; however, 5-nitrouracil inhibited growth stimulated by folic acid but not by thymine, while 5-bromouracil inhibited growth stimulated by thymine but not by folic acid. Hitchings *et al.* (4) accordingly suggested that "the two nutrients act, in some fashion, as alternative rather than as two components of an anabolic system." Hall (5) has also questioned the suggestion of Stokes (3) and, as the result of data showing synergistic growth-promoting effects of folic acid and thymine on *Streptococcus faecalis* R, has indicated that "it thus seems likely that the thymine may actually be a precursor of folic acid or that thymine is participating in some alternative metabolic path."

In the present investigation, a folic acid analogue which competitively prevents the functioning of folic acid in *Lactobacillus casei* has been employed in order to determine the relationship of purines and thymine to folic acid. Application of the techniques of *inhibition analysis* gave results which indicate that folic acid functions as a coenzyme, or in the formation of a coenzyme, necessary for the biosynthesis of purines and thymine or their equivalents.

EXPERIMENTAL

Source of Materials—The folic acid used in these experiments was synthetic pteroylglutamic acid (folvite, folic acid powder) obtained from the Lederle Laboratories Division, American Cyanamid Company. The folic acid antagonist, a methyl-substituted pteroylglutamic acid prepared from α,β-dibromobutyraldehyde by the folic acid synthesis previously described (6), was obtained through the courtesy of Dr. E. L. R. Stokstad. Competitive inhibition of the functioning of folic acid by this antagonist

Table I

Comparison of Effects of Purines on Toxicity of Methylfolic Acid

Test organism, *Lactobacillus casei;* incubated 72 hours at 37°.

Purine	Methylfolic acid*	Galvanometer readings†	
		Without added thymine	With added thymine (30 γ per 10 cc.)
100 γ per 10 cc.	*γ per 10 cc.*		
None	0	75.5	81.5
	1	79.0	80.5
	3	3.0	8.0
Guanine	0	89.5	90.0
	3	86.0	89.5
	10	5.0	86.0
	30		80.0
	100		12.0
Adenine	0	90.0	91.0
	3	86.5	90.0
	10	10.0	87.5
	30	3.0	84.5
	100		29.0
Xanthine	0	91.0	91.0
	3	83.0	91.0
	10	5.0	89.0
	30	3.0	85.5
	100		20.0
Hypoxanthine	0	91.0	91.0
	3	88.5	91.0
	10	5.0	90.0
	30	3.0	82.0
	100		27.0

* In the presence of 0.03 γ of folic acid per 10 cc.

† A measure of culture turbidity; an opaque object reads 100, distilled water 0.

has been previously demonstrated by Franklin, Stokstad, Belt, and Jukes (7). The antagonistic action of the other stereoisomer termed "methylfolic acid" has also been reported (8). The term methylfolic acid as used throughout this paper applies to the analogue of Franklin *et al.* (7).

Testing Methods—The methods of biological testing have been previously described (9). The medium (9) was modified by the omission of folic acid, purines, and uracil and by the addition of 1 mg. of asparagine, 10 γ of pyridoxine hydrochloride, and 1 γ of *p*-aminobenzoic acid per 10 cc. Culture tubes were inoculated with washed cells from actively growing cultures (16 to 24 hours old) of the test organism grown in yeast extract-peptone medium. Time and temperature of incubation are given in Tables I to III.

TABLE II

Effect of Hypoxanthine on Toxicity of Methylfolic Acid

Test organism, *Lactobacillus casei*; incubated 66 hours at 37°.

Methylfolic acid	Folic acid	Galvanometer readings	
		Without added hypoxanthine	With added hypoxanthine (100 γ per 10 cc.)
γ per 10 cc.	*γ per 10 cc.*		
0	0.01	81.0	88.5
0.3	0.01	79.0	89.0
1.0	0.01	3.5	85.5
3.0	0.01		4.0
0	0.1	85.5	89.5
1	0.1	84.0	90.0
3	0.1	6.0	90.0
10	0.1		6.0
0	0.3	82.0	90.5
3	0.3	82.5	90.0
10	0.3	4.0	77.0
30	0.3		6.0
0	1.0	75.0	90.0
10	1.0	70.5	90.5
30	1.0	4.0	68.5
100	1.0		5.0
Antibacterial index		30 *Ca.*	100 *Ca.*

Results

As indicated in Table I, thymine had no effect on the toxicity of methylfolic acid for *Lactobacillus casei* in the absence of exogenous purines. Addition of guanine, adenine, xanthine, or hypoxanthine to the medium necessitated an increased concentration of methylfolic acid in order to inhibit growth of the organism. In separate tests, this increase was never greater than 3-fold and often only about 2-fold. Hypoxanthine gave more consistent results than did the other purines. When one of the purines was added to the medium, the addition of thymine further prevented the toxicity of the analogue; so that the concentration necessary to inhibit growth in this case increased about 10- to 30-fold.

TABLE III

Effect of Thymine and Hypoxanthine on Toxicity of Methylfolic Acid

Test organism, *Lactobacillus casei;* incubated 60 hours at 37°.

Methylfolic acid	Folic acid	Galvanometer readings		
		Without added reversing agent	With added hypoxanthine (100 γ per 10 cc.)	With added hypoxanthine (100 γ per 10 cc.) and thymine (30 γ per 10 cc.)
γ per 10 cc.	*γ per 10 cc.*			
0	0.03		91.5	90.0
1	0.03		91.5	
3	0.03		21.0	
10	0.03		3.0	90.0
30	0.03			12.0
100	0.03			4.5
0	0.1		92.0	91.0
3	0.1		92.5	
10	0.1		5.0	
30	0.1			71.5
100	0.1			13.0
300	0.1			5.0
0	0.3	84.5	92.5	91.5
3	0.3	87.0	92.0	
10	0.3	5.0	81.0	
30	0.3		4.0	
100	0.3			39.0
300	0.3			15.0
1000	0.3			10.0
3000	0.3			29.0*
0	1.0	86.0	91.5	92.0
10	1.0	83.5	91.5	
30	1.0	4.0	28.5	
100	1.0		4.0	93.0
300	1.0			25.0
1000	1.0			14.0
3000	1.0			26.0*
Antibacterial index..........		30 *Ca.*	100 *Ca.*	1000 *Ca.*

* Some of the turbidity was due to precipitation, but slight growth was visible.

The effect of hypoxanthine on the toxicity of methylfolic acid is shown in Table II. From concentrations of 0.1 to 1.0 γ per 10 cc. of folic acid, the ratio of analogue to metabolite just necessary for maximum inhibition of growth (antibacterial index) was about 30 in the absence of added hypoxanthine but increased to about 100 in the presence of added hypoxanthine. The ratio of methylfolic to folic acid just necessary to inhibit growth changed only 3-fold on increasing the folic acid concentration from 0.01 to 0.1 γ per 10 cc. This deviation from the constant ratio usually obtained in other analogue-metabolite growth inhibitions may be explained by the assumption that enzyme saturation is not attained at such low concentration levels.

In Table III, the effect of thymine on the toxicity of methylfolic acid after addition of hypoxanthine to the medium is shown. Though inactive in a medium containing no purines, thymine added to a medium containing purines prevented the toxic action of methylfolic acid over a 10-fold increase in concentration. Thus, the antibacterial index was 30 in the absence of purines and thymine, 100 in the presence of purines, and approximately 1000 in the presence of purines and thymine. In the last case, the growth of the organism was not completely inhibited, particularly at higher concentrations of folic acid. In separate tests, there were indications that methylfolic acid was capable of diminishing the growth only to a certain level; for example, to that corresponding to a turbidity reading of 15 to 20. However, the inhibition of growth was sufficient to show that the point of maximum inhibition was dependent upon a ratio of methylfolic acid to folic acid of about 1000 to 3000 in all cases.

In separate tests, inhibition of growth of *Lactobacillus casei* with methylfolic acid in the medium containing no purines did not result in the accumulation of the amine which is formed during sulfonamide inhibition of a number of bacteria (10) and which has been identified as 5(4)-amino-4(5)-imidazolecarboxamide (11).

DISCUSSION

The term product inhibition index has been defined as the molar ratio of analogue to metabolite at which the rate of synthesis of the product of the inhibited enzymatic reaction is the limiting process for growth and is reduced to such an extent as to prevent growth of the organism in a medium not containing this product (12). This term can also be extended to instances in which the biosynthesis of a coenzyme is prevented by an analogue of a vitamin, and, as a result of reducing the rate of coenzyme synthesis, an enzymatic reaction involving this coenzyme is deprived of its coenzyme because of a greater affinity of other apoenzymes for the same coenzyme. Thus, an exogenous supply of the product of such an enzymatic reaction would allow growth to take place at a lower rate of coenzyme synthesis.

Since the ratio of the analogue to the metabolite determines the rate of conversion of metabolite to coenzyme (13), addition of the product which allows growth to occur at a lower rate of synthesis will result in an increase in the ratio of analogue to metabolite necessary just to prevent growth.

The competitive inhibition of folic acid by methylfolic acid could involve either a direct combination of methylfolic acid with an enzyme of which folic acid is a coenzyme or, more likely, a combination of methylfolic acid with an enzyme involved in the conversion of folic acid to a coenzyme. In either event, the term product inhibition index as outlined above can be applied. Specifically, the purine inhibition index of *Lactobacillus casei* with regard to the inhibition by methylfolic acid may be interpreted to be approximately 30, the thymine inhibition index approximately 100, and the inhibition index of another product, as yet not investigated, 1000 to 3000. These inhibition indices are merely the ratio of analogue to metabolite at which the biosyntheses of these products become the immediate limiting factors for growth.

In the presence of both a purine and thymine, growth of *Lactobacillus casei* was reduced to a low level by the analogue, but complete inhibition of growth was often not attained. Such results could be explained by assuming that the analogue has some activity in promoting growth in the presence of thymine and purines. Similar results have been obtained with pantothenic acid analogues (14). However, in view of the ability of *Lactobacillus casei* to grow in the absence of folic acid under these conditions, the possibility of a very slow rate of synthesis of folic acid seems likely, and folic acid synthesized by the organism may not be antagonized readily by the analogue. For example, the growth of yeast stimulated by β-alanine was not antagonized by N-pantoyltaurine, while that stimulated by pantothenic acid was prevented by the analogue (15).

Demonstration of the presence of folic acid in the cells of *Lactobacillus casei* grown in thymine and purines (3) would be exceedingly difficult, since the requirement for folic acid under these conditions probably would be only a small fraction of the requirement under the usual assay conditions with a medium not containing thymine. Furthermore, if folic acid is synthesized by the organism under these conditions, it may be converted to a coenzyme entirely different from that involved in purine or thymine synthesis and would thus escape detection.

The synergistic action of thymine and folic acid in promoting growth (5), in a medium containing purines, is most readily explained by the sparing effect of suboptimal amounts of thymine on the quantity of folic acid necessary for growth of *Streptococcus faecalis* R, since exogenous thymine in such amounts would allow a decreased rate of thymine synthesis for the same growth rate and a consequent decrease in the folic acid requirement.

The results of Hitchings *et al.* (4) with thymine analogues cannot be interpreted accurately in the absence of data indicating that these substances are specifically competitive with thymine itself rather than with related metabolites. For example, in a medium containing purines, 5-nitrouracil, which prevented growth of *Lactobacillus casei* stimulated by folic acid, does not competitively inhibit the functioning of thymine as such, since growth stimulated by thymine was not prevented by the inhibitor. The results obtained with 5-bromouracil may be interpreted by assuming a competitive prevention of the conversion of thymine to a conjugated derivative which is the immediate product of folic acid functioning. In such a case, growth stimulated by folic acid would not be inhibited by the thymine analogue, since thymine as such may never be involved in the biosynthesis. However, another type of action known to occur with pantothenic acid analogues would offer sufficient explanation for the observations of Hitchings *et al.* (4). The metabolite when synthesized by the organism is not antagonized by all of the analogues which are effective when biosynthesis is not occurring in the cell. This type of phenomenon will be discussed in more detail in later papers.

Although our results do not preclude the possibility that thymine may also function in the biosynthesis of folic acid, the most important action of thymine as well as of purines in preventing the toxic action of methylfolic acid appears to be that of supplying the product of an inhibited enzymatic reaction involving folic acid, since the effect of thymine as well as purines in preventing the toxicity of the analogue was not "diluted out" at ratios of inhibitor to metabolite involving high concentrations of folic acid (16).

The inhibition of growth of *Lactobacillus casei* with sulfanilamide and its reversal with *p*-aminobenzoic acid and with purines (17) indicates that the functions of *p*-aminobenzoic acid in purine synthesis (10–12) probably are not carried out totally by folic acid, which is required by the organism and is not replaced by *p*-aminobenzoic acid under the testing conditions employed.

SUMMARY

The antibacterial index obtained with a folic acid analogue, methylfolic acid, was approximately 30 for *Lactobacillus casei* in the absence of exogenous purines and pyrimidines. Addition of adenine, guanine, hypoxanthine, or xanthine to the growth medium resulted in an increased antibacterial index of approximately 100. Thymine alone had no effect on the toxicity of the analogue, but, in the presence of purines, the antibacterial index was increased to 1000 or slightly higher. These results indicate that folic acid functions in the biosynthesis of purines and thymine or their equivalents.

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PHOSPHATASE ACTIVITY IN SUCKLING PIGS

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A previous publication (1) described a condition in young pigs which is characterized pathologically by changes in the liver. Studies on the alkaline serum phosphatase activity of these pigs have indicated that there is no correlation between the hepatic changes observed and the phosphatase activity. Since a survey of the literature did not disclose a report on phosphatase activity in suckling pigs, such studies were made on seven litters that appeared normal at birth.

Methods

Blood samples were taken from 50 pigs at 0, 5, and 14 days of age and at weekly intervals thereafter until the pigs were 56 days old. All samples were obtained from the anterior vena cava by means of a syringe and needle. The samples were allowed to clot, placed at 4° overnight, and centrifuged. The serum was removed by decantation.

Alkaline serum phosphatase activity was determined by the method of Bessey, Lowry, and Brock (2) with 0.1 ml. of serum. By this test the amount of *p*-nitrophenol liberated by the action of the enzyme phosphatase upon the substrate *p*-nitrophenyl phosphate is measured. 1 mM unit is the degree of phosphatase activity which will liberate 1 mM of nitrophenol per liter of serum per hour, and is approximately equivalent to 1.8 Bodansky units.

Results

The results are summarized in Fig. 1, which shows a comparison of alkaline serum phosphatase activity and the growth rate of suckling pigs. As indicated in Fig. 1, phosphatase activity is very high in the new-born pig, but falls considerably by the 5th day. Thereafter a gradual decline in phosphatase activity occurs up to the 4th week, after which time the activity is fairly constant.

Of the seven litters studied, four consisted of apparently normal pigs as indicated by the average weaning weight of 32 pounds. The other three litters were less thrifty, as indicated by their average weaning weight of only 20 pounds. Comparison of phosphatase activity in these two groups showed no significant differences; therefore the data for all litters were lumped for this report. Evidently the factors responsible for the unthrifty condition in these three litters were not related to phosphatase activity.

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The variations in individual phosphatase activity are not indicated in Fig. 1. Actually, considerable variation was encountered, as is shown by Table I, which gives the average phosphatase values, m, and the standard deviations, s, in mM units, and the coefficients of variation, m/s, in per cent. The greatest variations occurred at the 14 and 21 day periods, indicating that great differences among individuals occur at this time.

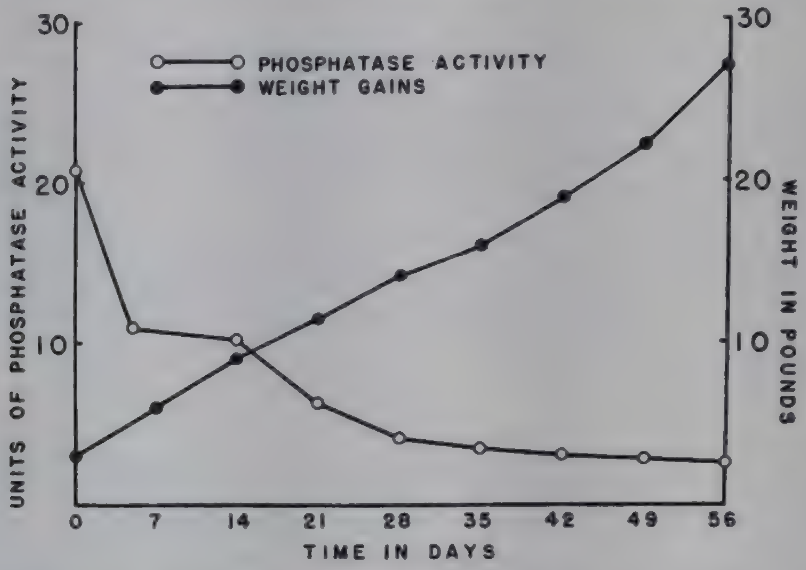

FIG. 1. Comparison of alkaline serum phosphatase activity and rate of growth in suckling pigs. Phosphatase activity is indicated in mM units (1 mM of nitrophenol liberated from p-nitrophenyl phosphate per liter of serum per hour).

TABLE I

Variation in Phosphatase Activity among Suckling Pigs

Age at time of tests	No. of samples	Mean, m	Standard deviation, s	Coefficient of variation, $\frac{m}{s}$
days		*mM units*	*mM units*	*per cent*
0	50	20.9	8.93	42.7
5	49	11.0	2.78	25.2
14	48	10.3	9.04	87.7
21	46	6.3	5.73	90.9
28	47	4.1	0.98	23.9
35	47	3.5	1.24	35.4
42	47	3.2	0.81	23.1
49	47	2.9	1.0	34.4
56	47	2.6	1.08	41.5

SUMMARY

Alkaline serum phosphatase activity is reported for suckling pigs from 0 to 56 days of age. Values were quite high at birth, a mean of 20.9 mM nitrophenol units, but were reduced approximately one-half by the 5th day. Phosphatase activity was shown to level off at about 28 days of age.

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FACTORS AFFECTING THE AVAILABILITY OF LYSINE IN HEAT-PROCESSED CASEIN

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It has been demonstrated that the nutritive value of casein is considerably impaired by heating in the dry state at elevated temperatures (1, 2). Supplementation of the diet with lysine, however, effectively restores its nutritive quality. The adverse effect of heating upon casein could not be attributed to destruction of its lysine content, since analysis of acid hydrolysates of heat-processed casein by chemical and enzymic procedures resulted in complete recoveries of this amino acid (3, 4). It has been postulated (3, 5) that in heating casein a peptide linkage is formed between the ϵ-amino group of lysine and a free carboxyl group of one of the dicarboxylic acids, and that this complex is resistant to enzymic digestion. Eldred and Rodney (3) found decreased availability of the lysine in enzymic digests of heated casein, using the specific enzyme lysine decarboxylase for the analyses.

The hypothesis of Melnick, Oser, and Weiss (6), bearing on the relation between the biological value of a protein and the rate of enzymic liberation of its amino acids, was based upon studies of the effects of heat processing on a variety of protein-containing materials. These authors stated that "for optimum utilization of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation." Papers supporting this concept have recently appeared (7, 8).

In the present study, *in vitro* digestibility techniques and microbiological assays were employed to determine factors affecting the availability of lysine in heat-processed protein, with casein as a prototype.

Methods

Samples of casein[1] were heated in a drying oven at 150° for periods of 1.5 and 5 hours. These preparations and the original sample were used throughout the current study. The lysine content of the proteins was determined microbiologically (following acid hydrolysis) by the procedure of Stokes *et al.* (9), with *Streptococcus faecalis* R as the test organism. All values were calculated to the pure protein basis, 15.4 per cent nitrogen.

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* Submitted in partial fulfilment of the requirements for the degree of Master of Science at New York University.

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[1] Obtained from the S. M. A. Corporation, Chagrin Falls, Ohio.

In order to evaluate the *extent* to which lysine could be liberated from casein for microbiological utilization, samples of casein (equivalent to 3.00 gm. of protein) were incubated at 37° for 24 hours in 110 ml. of an 0.082 N HCl solution containing 0.30 gm. of pepsin;[2] this was followed by incubation of the solution at pH 8.3 at the same temperature for an additional 96 hours with 0.30 gm. of pancreatin.[3] The pepsin was inactivated by heating the test solution, at pH 7, in a boiling water bath prior to adding the pancreatin. The degree of hydrolysis of the resulting digests was determined by formol titration (6); the degree of liberation of lysine for microbiological utilization was estimated by the same assay procedure (9) employed in analyzing the acid digests, with the modification that the samples were not subjected to acid hydrolysis. Thus, the apparent lysine values included both the free amino acid and the amino acid in peptide combinations utilizable by the microorganism.[4]

TABLE I

Lysine Content of Test Casein Samples

Casein sample	Lysine found*
	per cent
Unheated	8.1
Heated 1.5 hrs. at 150°	7.9
" 5 hrs. at 150°	7.6

* Determined microbiologically after complete acid hydrolysis; values calculated to the pure protein basis, 15.4 per cent nitrogen.

The *in vitro* procedure of Melnick *et al.* (6) for determining the susceptibility of proteins to enzymic digestion was modified to include measurement of the degree of hydrolysis after digestion of the proteins for 2 and 6 hours, as well as after 1, 2, and 5 days. Also, the available lysine content of the serial digests was determined microbiologically without removal of undigested protein.[5]

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[2] National formulary, Easton, 8th edition (1946).

[3] Pancreatin, 1:110. Obtained from the Pfanstiehl Chemical Company, Waukegan, Illinois.

[4] In all microbiological assays for lysine involving preliminary enzymic digestion of the samples, corrections were made for microbiological stimulatory complexes derived from enzyme materials.

[5] The quantity of trichloroacetic acid-precipitatable material was negligible after digestion of the protein for 1 day.

Results

It is apparent from the data in Table I that lysine was not destroyed to any appreciable extent during the heating of the casein sample.

TABLE II

*Enzymic Liberation of Lysine for Microbiological Utilization**

Casein sample	Degree of hydrolysis†	Microbiologically available lysine‡	Lysine utilized§
	per cent	*per cent*	*per cent*
Unheated	57	6.5	80
Heated 1.5 hrs. at 150°	51	5.9	75
" 5 hrs. at 150°	57	5.2	68

* Peptic digestion followed by pancreatic digestion (see the text).

† Estimated by formol titration.

‡ In the final enzymic digest, calculated to the original protein basis.

§ Per cent of total lysine in casein samples.

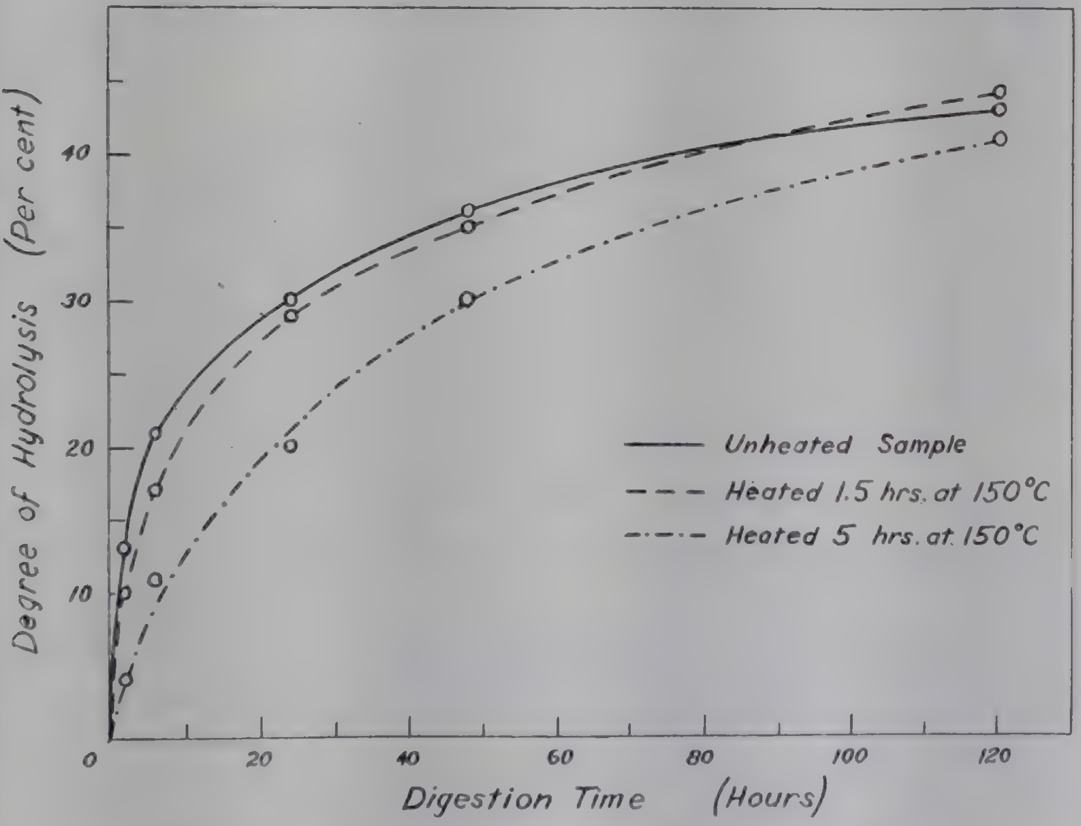

FIG. 1. Rate of *in vitro* pancreatic hydrolysis of casein

In Table II are presented the microbiologically available lysine values of the casein samples following maximal enzymic digestion (peptic and pancreatic). It is apparent that both the degree of protein hydrolysis and the apparent lysine content were not greatly affected by subjecting the casein sample to the heat treatment. There is, however, a suggestion that extending the period of heating reduced the available lysine content to a small degree.

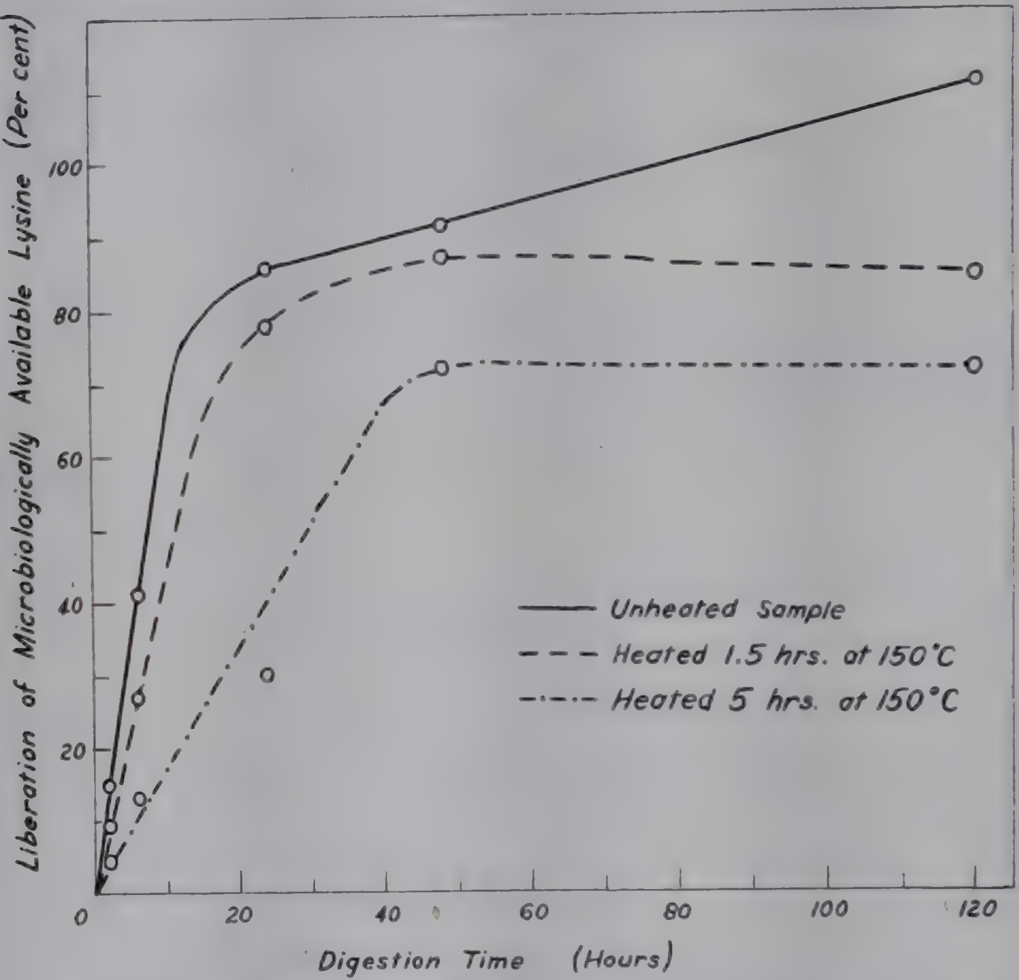

FIG. 2. Rate of *in vitro* pancreatic liberation of microbiologically available lysine from casein.

In contrast to the above findings, striking differences in the *susceptibility* of the heated and unheated casein samples to *in vitro* pancreatic digestion were noted.[6] The data plotted in Fig. 1 indicate that the heated protein samples were digested at significantly slower rates than the unheated control sample, and that the rates of liberation of formol-titratable nitrogen varied inversely with the processing time. The differences were of greatest relative magnitude during the early phase of hydrolysis. The ultimate degree of digestion of both the heated and unheated products eventually approached the same value, in agreement with the results of the serial enzymic digestion experiments (see Table II).

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[6] The use of suboptimal quantities of pancreatin permits the digestion to proceed for several days at 37°, thus retarding the liberation of the amino acids to reveal differences in the rate of digestibility (6).

The differences in the *rate* of liberation of the available lysine from the casein samples was even more striking. Reference to Fig. 2 shows that microbiologically available lysine was liberated from the heat-treated protein by pancreatin at a much slower rate than from the unheated product. The curves relating liberation of available lysine to time are linear up to a point at which they form a plateau. The relative slopes of the linear portions of the curves are as follows: 1, arbitrarily assigned to the control unheated sample; 0.66, casein heated 1.5 hours; and 0.25, sample heated 5 hours. Thus, substances with lysine activity were released initially from the unheated protein at a rate 4 times as great as from the sample submitted to prolonged heating, and 1.5 times as rapidly as from the intermediate preparation.

TABLE III

Influence of Further Enzymic Hydrolysis of Partially Digested Casein Samples on Release of Amino Nitrogen and Microbiologically Available Lysine

Casein sample	Preliminary pancreatic digestion			Subsequent serial enzymic digestion*	
	Time	Protein hydrolysis†	Microbiologically available lysine‡	Protein hydrolysis†	Microbiologically available lysine‡
	hrs.	*per cent*	*per cent*	*per cent*	*per cent*
Unheated	24	30	7.0	47	6.7
	48	36	7.4	49	6.9
	120	43	8.9	47	6.1
Heated 1.5 hrs. at 150°	24	29	6.2	49	5.3
	48	35	6.9	48	5.4
	120	44	6.6	46	5.2
Heated 5 hrs. at 150°	24	20	2.4	48	4.4
	48	30	5.5	46	4.1
	120	41	5.4	46	4.4

* Peptic digestion followed by pancreatic digestion (see the text).

† Estimated by formol titration.

‡ In the final enzymic digest calculated to the original protein basis.

Further peptic and pancreatic treatment of the 1, 2, and 5 day pancreatic digests, already containing a major portion of the lysine in microbiologically available form, yielded solutions with apparently lower lysine contents, despite increases in the degree of protein hydrolysis. These data are presented in Table III.

DISCUSSION

The data presented in this report indicate that in enzymic digests of casein the microbiologically available lysine contents are greater than those expected from the degree of protein hydrolysis effected. Undoubtedly, the test organism, *Streptococcus faecalis* R, is capable of utilizing lysine bound in peptide linkages. The factor of strepogenin stimulation (10) was eliminated in the current studies by permitting the test organism to grow in the assay medium for a period of 3 days prior to titrimetric evaluation. (The polypeptide strepogenin is known to stimulate the growth of microorganisms only during their initial growth phases. For this reason, assays for this factor are usually completed within the first 24 hours of incubation.)

The differences in the content of microbiologically available lysine in the final enzymic digests of heated and unheated casein are insufficient to account for the reduced biological value of the heated protein. The results of studies of the rate of liberation of lysine for microbiological utilization provide a plausible explanation for the impairment of the biological value. Thus, during the early periods of *in vitro* digestion, lysine is liberated from the more strenuously heated sample at a rate only one-fourth of that noted in the tests with the unheated casein. When the digestion is continued until maximal, constant values are obtained; the microbiologically available lysine content of the heated casein is fully three-fourths of that of the control sample. Since, for optimal utilization, the essential amino acids must be absorbed *in vivo* at rates permitting mutual supplementation (6–8), the *rate* of release of lysine from heat-processed casein is regarded as critical in determining the biological value.

Data have been obtained in the current studies suggesting that lysine, in certain peptide combinations, is stimulatory for microbiological growth to a greater degree than the free amino acid itself. Thus, it will be noted that in the 5 day pancreatic digest of the control, unheated casein sample, an apparent value of 110 per cent of the theoretical (lysine found after acid hydrolysis) was obtained (see Fig. 2). Furthermore, on extending the enzymic digestion of the samples, decreases in the microbiologically available lysine content were noted despite increases in formol-titratable nitrogen values[7] (see Table III). It is concluded that during the period of

[7] The one exception to this statement in the series of tests referred to was the digest which exhibited a marked increase in degree of protein hydrolysis on extending the hydrolysis. It is believed that in this case the release of more available lysine effected a microbiological response sufficiently great to mask the loss of microbiological activity resulting from the hydrolysis of lysine peptide combinations originally present.

prolonged enzymic digestion the highly active lysine complexes were degraded, and so apparently lower lysine values were obtained. The instability of the polypeptide strepogenin in aqueous solutions held under similar extended periods of storage, is known.

SUMMARY

1. An *in vitro* study was conducted to determine the factors responsible for the decreased availability of lysine in heat-processed protein, with casein as a prototype.

2. Dry heating at 150° decreased the *rate* of *in vitro* pancreatic hydrolysis of casein appreciably, but did not significantly affect the ultimate *degree* to which the protein was hydrolyzed.

3. The *rate* at which lysine was released from casein by pancreatic digestion, as measured by availability of the amino acid to *Streptococcus faecalis* R, was decreased to a great extent by baking the protein. This treatment also decreased the ultimate degree to which it was possible to liberate lysine for microbiological utilization, though to a less significant degree.

4. The most significant differences between the rates of pancreatic liberation of lysine from heated and unheated casein were noted in the early stages of protein hydrolysis. The magnitude of these differences was sufficient to explain the poor biological values noted by others on feeding heated casein to test animals. It is concluded that, in heated casein, the lysine is liberated at a rate too slow to allow effective supplementation of the other amino acids absorbed earlier in the process of digestion.

5. *Streptococcus faecalis* R utilizes lysine in certain peptide combinations more effectively than it does the free amino acid itself.

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STUDIES ON INTERMEDIARY STEROID METABOLISM

I. ISOLATION OF Δ^5-ANDROSTENE-3(β),17(α)-DIOL AND Δ^5-ANDROSTENE-3(β),16(β),17(α)-TRIOL FOLLOWING THE INCUBATION OF DEHYDROISOANDROSTERONE WITH SURVIVING RABBIT LIVER SLICES

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Until recently, most studies of steroid metabolism have centered about the isolation and characterization either of compounds normally found in the urine (1–7) or of those appearing as the result of the administration of known substances (8). These investigations have been supplemented by various studies of the steroid content of pathologic urine, particularly that obtained from patients with hyperactive adrenal cortical lesions (9–13). Since such patients often excrete large amounts of steroids, the task of isolation is considerably facilitated, and, in addition, the information obtained serves as a valuable diagnostic aid. Prominent among the ketosteroids excreted by patients with adrenocortical tumors is dehydroisoandrosterone, which may appear in amounts of several hundred mg. daily. It is evident that this compound should hold considerable interest as a possible intermediate in steroid metabolism.

In an investigation of the possible rôle of dehydroisoandrosterone as an intermediate in the conversion of testosterone to androsterone in human subjects, Dorfman and Hamilton (14) noted a marked increase of the urinary androgen level following administration of this substance but did not isolate any products. Munson, Gallagher, and Koch (15) examined the urine of a woman who had been given dehydroisoandrosterone acetate parenterally. Since they were able to isolate dehydroisoandrosterone in an amount corresponding to 25 per cent of the conjugated neutral 17-ketosteroids, they concluded that the greater part of the administered steroid was not metabolized further but was excreted unchanged. However the other fractions were not investigated. Later, Mason and Kepler (16) demonstrated the excretion of significant amounts of androsterone, etiocholan-3(α)-ol-17-one, and Δ^5-androstene-3(β),17(α)-diol following the administration of dehydroisoandrosterone to patients whose normal ketosteroid excretion was very low. It is of interest to note in this connection that Miller and Dorfman (17) were able to isolate small amounts of dehydroisoandrosterone from the urine of guinea pigs after the subcutaneous injection of Δ^5-androstene-3(β),17(α)-diol and thus further established a relationship between dehydroisoandrosterone and androstenediol.

As a means of obtaining additional information concerning the metabolic pathways involved, it appeared logical to investigate the fate of certain representative ketosteroids when incubated with various tissues. To date, *in vitro* studies of steroid metabolism have been limited almost entirely to those on the inactivation of testosterone (18–22), progesterone (23), and estrogens (24) by liver tissue. However, no attempts were made in these experiments to recover any of the metabolic products. More recently, Clark and Kochakian (25) isolated *cis*-testosterone and Δ^4-androstene-3,17-dione after the incubation of testosterone with rabbit liver slices. It is the purpose of this paper to describe the isolation and characterization of the metabolic products formed when dehydroisoandrosterone[1] was incubated with surviving rabbit liver slices under aerobic conditions.

Since the requirements of the enzyme systems involved in steroid synthesis or degradation are largely unknown, it seemed desirable in these initial experiments to simplify the system employed. Accordingly, neither glucose nor known cofactors were added. Further simplification was achieved by adding the steroid to the system in the form of the readily soluble sodium salt of its hemisuccinate, thus eliminating the necessity for employment of serum or other dispersing agents. Preliminary experiments had demonstrated that the rate of hydrolysis of this ester in liver is rapid and could not be a limiting factor.

Methods

Incubation—Slices were prepared from well chilled rabbit livers which had been removed immediately after decapitation and bleeding of the animal. These were collected in chilled, aerated Krebs and Eggleston's phosphate saline buffer (26), washed twice with the same solution, drained and blotted free of buffer, weighed, and transferred to a 2 liter wide mouth bottle containing buffer and sodium dehydroisoandrosterone succinate. The latter was prepared by the addition of dilute sodium hydroxide to a suspension of the hemisuccinate in 10 ml. of water until solution was achieved; the solution was then added to the buffer. The bottle was suspended on its side in a mechanical shaker that provided adequate to and fro agitation and which included a device for maintaining the temperature at 38–40°. In order to avoid undue fragmentation of the slices, oxygen was passed over, rather than into, the contents of the bottle. The rate of agitation, the depth of the liquid, and the flow of oxygen were so regulated as to provide ample oxygenation and free movement of the suspended slices. The liver slices prepared for these experiments were of such a size and thickness that roughly one-half of them remained intact through the incubation period.

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[1] The authors wish to thank Dr. E. Oppenheimer and Dr. C. R. Scholz, of Ciba Pharmaceutical Products, Inc., for a generous gift of dehydroisoandrosterone.

Protein Precipitation and Extraction—At the end of the incubation period 20 ml. of concentrated hydrochloric acid were added to the incubation bottle and the contents were then poured into 2 liters of cold, neutral acetone. The acid-acetone mixture effectively precipitated the proteins and assured the solution of whatever unhydrolyzed succinate may have been present. After the still intact slices and tissue débris had settled, the acetone solution was filtered with suction and then, in order, the tissue was ground thoroughly with sand, replaced on the filter, and repeatedly washed with ten 200 ml. portions of boiling neutral acetone. The acetone solutions were combined and either concentrated immediately or placed in the cold for future attention. Since it was found difficult to neutralize acid-acetone solutions accurately, no attempt was made to neutralize the hydrochloric acid until after the acetone was removed under reduced pressure. In those experiments in which the steroid was incubated in portions, it was found advantageous to neutralize and combine the several aqueous residues as they appeared and to store the combined solutions in the cold until extracted. The further processing of these residues is outlined in the accompanying diagram.

Ethylene dichloride appeared to be the solvent of choice for extraction of the aqueous residues, largely because it did not form emulsions even in alkaline solution. The combined neutral aqueous residues were diluted with an equal volume of water and adjusted to pH 9 by addition of sodium carbonate. The "free" steroid fraction was then obtained by extraction with ethylene dichloride. A "conjugated"[2] steroid fraction was obtained by further extraction after acidification of the aqueous solution.

Fractionation Procedure—The petroleum ether fractionation, as shown in the diagram, served to concentrate the greater part of the products formed in the petroleum ether-insoluble phase, while at the same time it removed all but traces of cholesterol, fat, and colored materials. It was carried out by warming the dried "free" steroid residue with 50 ml. of petroleum ether, and, after filtration, the insoluble fraction was washed with another portion of the solvent. The dried petroleum ether-insoluble fraction was usually white, crystalline, or partly crystalline, and usually accounted for from 75 to 80 per cent of the added steroid. This fractionation was not applicable to the "conjugated" fraction, and has not been successful with either fraction in those cases in which compounds other than dehydroisoandrosterone were incubated. Thus, no petroleum ether-insoluble fraction was obtained after the incubation of androsterone.

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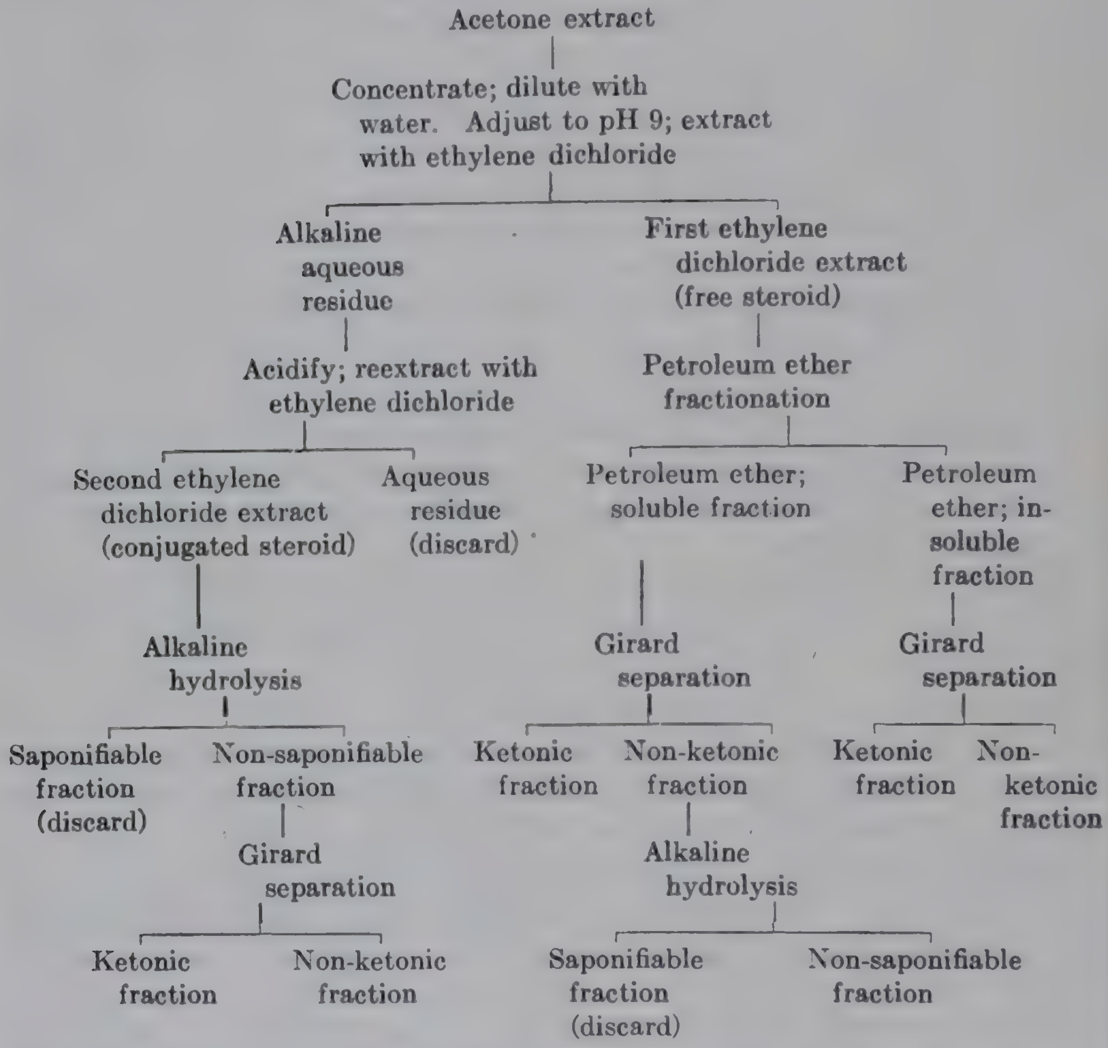
[2] It is to be expected that this procedure would not effectively recover any steroid conjugated with sulfuric or glucuronic acids. Accordingly, this fraction was composed largely of unhydrolyzed succinate. The results of Samuels and his associates (22) with testosterone indicate that little conjugation took place in the system that they employed.

As indicated in the diagram, the treatment of the "free" steroid fraction with Girard and Sandulesco's Reagent T (27) was carried out after rather than before the petroleum ether fractionation. This procedure was adopted because it was found more difficult to accomplish a clean cut separation when the entire ethylene dichloride extract was used. Since the greater part of the cholesterol, cholesterol esters, and fat was concentrated in the petroleum ether-soluble, non-ketonic moiety, this fraction was saponified and the non-saponifiable fraction was recovered and chromatographed. It was not possible to accomplish any separation by treatment of the crude "conjugated" fraction with Girard and Sandulesco's Reagent T. It was

Fractionation Procedure


Acetone extract
Concentrate; dilute with water. Adjust to pH 9; extract with ethylene dichloride
Alkaline aqueous residue
First ethylene dichloride extract (free steroid)
Acidify; reextract with ethylene dichloride
Petroleum ether fractionation
Second ethylene dichloride extract (conjugated steroid)
Aqueous residue (discard)
Petroleum ether; soluble fraction
Petroleum ether; insoluble fraction
Alkaline hydrolysis
Girard separation
Girard separation
Saponifiable fraction (discard)
Non-saponifiable fraction
Ketonic fraction
Non-ketonic fraction
Ketonic fraction
Non-ketonic fraction
Girard separation
Alkaline hydrolysis
Ketonic fraction
Non-ketonic fraction
Saponifiable fraction (discard)
Non-saponifiable fraction

necessary to hydrolyze the entire fraction and then to examine the nonsaponifiable portion for ketonic materials.

Chromatographic analysis was carried out on columns of aluminum oxide (Fisher's alumina for chromatographic analysis, 80 to 200 mesh). The amount of alumina was 30 to 40 times the weight of the mixture to be examined. Redistilled analytic reagent carbon tetrachloride was used as the solvent for the ketonic fractions. The columns were developed and the compounds eluted by washing with appropriate mixtures of absolute alcohol and carbon tetrachloride as described by Callow and Callow (3). Redistilled thiophene-free benzene was used as the solvent for the nonketonic fractions, and the columns were developed and the compounds eluted with mixtures of absolute alcohol and benzene. The last, most adherent non-ketonic fraction was removed by passing a 20:2:78 mixture, by volume, of pyridine, acetic acid, and ether over the column. The products obtained were further purified by repeated crystallization and were finally identified by their melting points, the melting points of mixtures, and elementary analyses.

Results

Three separate experiments were carried out by the procedures described. In each experiment the sodium dehydroisoandrosterone succinate was incubated in divided amounts and the several extracts were combined before processing. In Experiment 1 two 1000 mg. portions of dehydroisoandrosterone hemisuccinate (each equivalent to 790 mg. of free steroid) were incubated with liver slices for 3 hour periods, in Experiment 2 two 500 mg. samples of hemisuccinate (each equivalent to 395 mg. of free steroid) were incubated for periods of 6 hours, while in Experiment 3, three 635 mg. samples of hemisuccinate (each equivalent to 500 mg. of free steroid) were incubated for 6 hour periods. The weight of tissue slices used for each of the seven incubations varied from 100 to 135 gm. They were suspended in each instance in 250 ml. of buffer.

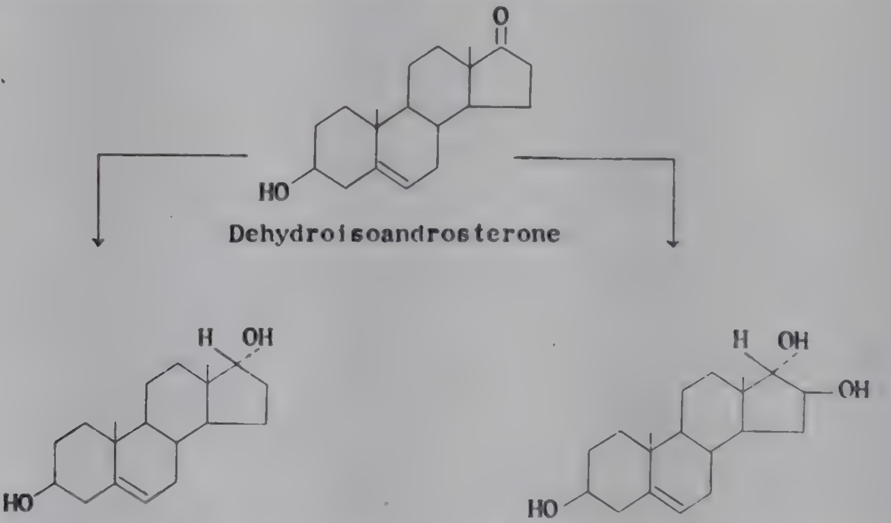
Table I lists the total weights of steroid and tissue used in each experiment, together with the weights of the major crude fractions. It is apparent that the petroleum ether fractionation served to divide the original crude extract in such a fashion that the weight of the petroleum ether-insoluble fraction roughly approximated the weight of steroid incubated. The weight of the crude "conjugated" fraction varied widely but in general was smaller than the weight of the crude "free" fraction.

In addition to unchanged dehydroisoandrosterone, two previously known crystalline compounds were isolated in all three experiments. These were Δ^5-androstene-3(β), 17(α)-diol, which was isolated in yields of 43, 59, and 69 per cent respectively, and Δ⁵-androstene-3(β),16(β),17(α)-triol, which was obtained in yields of 2.4, 8.9, and 5.0 per cent respectively. The structural relationships of these compounds are indicated in the accompanying formulas, and their distribution in the various crude fractions is indicated in Table II. It is evident that with the exception of Experiment 1 only a relatively small fraction of the added steroid hemisuccinate remained unhydrolyzed and appeared in the conjugated fraction. Both the diol and the triol were concentrated largely in the petroleum ether-insoluble non-ketonic fraction, while the dehydroisoandrosterone appeared in both the petroleum ether-soluble and the petroleum ether-insoluble ketonic fractions. The data on cholesterol are included to indicate the efficiency with which it was concentrated in the petroleum ether-soluble fraction.

TABLE I

Incubation of Dehydroisoandrosterone; Weights of Major Crude Fractions

Experiment No.	Total weight of dehydroisoandrosterone incubated	Total weight of tissue	Incubation period	Fractions obtained, mg.									
				Free steroid						Conjugated steroid			
				Total	Petroleum ether-soluble		Petroleum ether-insoluble			Total	Non-saponifiable		
					Total	Non-saponifiable	Total	Ketonic	Non-ketonic		Total	Ketonic	Non-ketonic
	mg.	*gm.*	*hrs.*										
1	1580	235	3	5350	3700	1090	1290	114	938	1610	721	364	320
2	790	250	6	3210	2530	593	612	35	516	2940	187	110	64
3	1500	300	6	5560	4260	1750	1210	0	1140	5670	1500	71	1380


Dehydroisoandrosterone

Δ⁵-Androstene-3(β),17(α)-diol

Δ⁵-Androstene-3(β),16(β),17(α)-triol

Besides the readily identifiable products, two other fractions were regularly obtained in small yield. The first of these was an α,β-unsaturated ketone which was isolated in crude form in about 1 per cent yield in all three experiments and ultimately crystallized in one instance. The α,β-unsaturated ketone group was indicated by an absorption maximum at 238 mμ. The melting point was not that of testosterone but approximated that of *cis*-testosterone. However, the melting point of the acetate was 174–176°, while the melting point of *cis*-testosterone acetate is 115–116°. The amounts of this compound available were insufficient for further characterization. The second, as yet unidentified fraction, a non-ketonic substance, was eluted from the column of alumina with a 90:10 (by volume) benzene-alcohol mixture. Attempts to crystallize the substance have not as yet been successful. It separated from methanol as balls with a gelatinous appearance. The over-all recovery of crystalline compounds including dehydroisoandrosterone was 68, 80, and 81 per cent in the three experiments.

TABLE II

Incubation of Dehydroisoandrosterone; Distribution of Compounds Isolated

Compound	Experiment No.	Fractions obtained, mg.				
		Free steroid				Conjugated steroid, non-saponifiable fraction
		Petroleum ether-insoluble		Petroleum ether-soluble		
		Ketonic	Non-ketonic	Ketonic	Non-ketonic	
Androstenediol	1	0	485	0	128	65
	2	0	378	0	55	35
	3	0	817	0	185	35
Androstenetriol	1	0	38	0	0	0
	2	0	45	0	25	0
	3	0	65	0	10	0
Dehydroisoandrosterone	1	75	0	0	0	280
	2	16	0	18	0	60
	3	0	0	96	0	0
Cholesterol	1	0	10	0	360	140
	2	0	17	0	251	212
	3	0	28	0	420	

The control experiments were of two types and proved to be of considerable interest. In two instances fresh rabbit livers were chilled, homogenized, poured into acid acetone, and processed according to the regular procedure. The second type of control experiment included the incubation of liver slices under the conditions used in the trial runs and differed only in that no extraneous steroid was added. In the first case no substances of interest were found, while in the second type of experiment a small ketonic fraction was obtained which on treatment with dinitrophenylhydrazine gave an immediate bright red precipitate, indicating the presence of an α,β-unsaturated ketone group. The substance or substances responsible have not as yet been isolated in pure form.

EXPERIMENTAL

All melting points were determined with the Fisher-Johns apparatus and are recorded as read.

Preparation of Dehydroisoandrosterone Succinate—Dehydroisoandrosterone was heated under a reflux for 2 hours with twice its weight of succinic anhydride and sufficient dry pyridine to achieve solution. After cooling, water and ice were added and the mixture was extracted with ether. The ether solution was washed with cold dilute sulfuric acid and then the succinate was extracted by washing the ether solution several times with dilute sodium carbonate solution. After washing the carbonate solution once with ether, the aqueous phase was acidified and reextracted with ether. The ether solution was washed with water, dried, and evaporated. The residue was taken up in methanol, from which the succinate readily separated. All traces of colored material were removed by recrystallization from the same solvent. A yield of 88 to 93 per cent of dehydroisoandrosterone hemisuccinate was obtained. It melted at 255–259°.

Isolation and Identification of Δ^5-Androstene-3(β),17(α)-diol—The androstenediol isolated appeared largely in the "free" petroleum ether-insoluble non-ketonic fraction. Its separation and purification were comparatively easy, since it was eluted from the column of alumina after the removal of cholesterol and before the recovery of androstenetriol. Usually, elution of this compound occurred when the concentration of alcohol in carbon tetrachloride reached 0.3 to 0.5 per cent. Final crystallization from methanol yielded colorless needles which melted at 176–179°. A mixture with an authentic specimen of Δ^5-androstene-3(β),17(α)-diol (m.p. 176–177°) melted at 176–177°. A sample treated with digitonin formed an immediate precipitate. The diacetate, prepared with the aid of acetic anhydride and pyridine, melted at 155°. The melting point was not depressed by admixture of authentic Δ^5-androstene-3(β),17(α)-diol diacetate.

Analysis of Diacetate—$C_{23}H_{36}O_4$. Calculated. C 73.36, H 9.64
Found. " 73.55, " 9.46

Isolation and Identification of Δ^5-Androstene-3(β),16(β),17(α)-triol—Androstenetriol was eluted from the column only after the application of the mixture of pyridine, acetic acid, and ether. It was usually accompanied by colored impurities which tended to inhibit crystallization, but ultimately it separated from methanol and was best recrystallized from this solvent. It melted at 265–268° and a mixture with an authentic specimen (m.p. 263–264°) melted at 260–266°. The triacetate melted at 184–185°, and a mixture with Δ^5-androstene-3(β),16(β),17(α)-triol triacetate (m.p. 183–185°) melted at 183–184°.

Analysis of Free Triol—$C_{19}H_{30}O_3 \cdot \frac{1}{2}CH_3OH$.[3]		
	Calculated.	C 72.72, H 9.95
	Found.	" 72.76, " 9.98

Isolation of α,β-Unsaturated Ketone—This substance accompanied dehydroisoandrosterone through the isolation procedure and was finally eluted from the column with or near the dehydroisoandrosterone fraction. It was separated from the known ketone by the use of digitonin, since under the conditions employed dehydroisoandrosterone alone formed a precipitate. The compound was crystallized from methanol and melted at 224–226°. The acetate melted at 175–176°. The compound formed a red amorphous precipitate with 2,4-dinitrophenylhydrazine. An alcoholic solution (5 mg. in 100 ml.) showed maximal absorption at 238 mμ.

Isolation of Dehydroisoandrosterone—Examination of the several ketonic fractions resulted in the isolation of dehydroisoandrosterone as the sole ketone in addition to the α,β-unsaturated ketone noted in the preceding section. Dehydroisoandrosterone was characteristically eluted from the column with a 0.1 to 0.2 per cent solution (by volume) of alcohol in carbon tetrachloride. It was recrystallized from ethyl acetate and then melted at 148–149°. A mixture with an authentic specimen of dehydroisoandrosterone (m.p. 146–148°) melted at 147–149°. The benzoate melted at 248–250°.

Isolation of Cholesterol—Although cholesterol was present in relatively large amounts, it did not interfere with the isolation of the other steroids, since the Girard separation and the petroleum ether fractionation served to remove all but traces of it from the important fractions. When present, it was eluted from the column with a 0.05 to 0.1 per cent solution of alcohol in benzene. The melting point was 149–150°. A mixture with an authentic specimen melted at 147–148°. After crystallization of the greater part of the cholesterol the mother liquors were further examined for compounds possibly eluted with the cholesterol. None were found.

Comment

The formation of Δ^5-androstene-3(β),17(α)-diol and Δ^5-androstene-3(β),16(β),17(α)-triol from dehydroisoandrosterone in the presence of liver tissue indicates that both of these substances can be regarded as products of its metabolism and serves again to emphasize the rôle of the liver as a site of steroid transformation. Although Δ^5-androstene-3(β),17(α)-diol has not yet been isolated from normal urine, it has been recovered from the urine of one patient after the administration of dehydroisoandrosterone (16) and from others with adrenal tumors (29, 30). Moreover, the reaction involved (namely, the reduction of the C-17 carbonyl group to a hydroxyl group) is paralleled by similar reactions in other compounds. The known conversions of estrone to estradiol and of progesterone to pregnanediol serve as familiar examples. It may be expected that in time this reaction will prove to be of rather general occurrence and importance in the intermediary metabolism of the 17-ketosteroids. In view of the high yield of Δ^5-androstene-3(β),17(α)-diol obtained, with the system described, it would seem of interest to test the occurrence of this reduction in liver mince and in cell-free systems. Such experiments are now in progress.

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[3] The retention of 0.5 mole of methanol is in accord with the observations of Hirschmann (28).

Δ^5-Androstene-3(β),16(β),17(α)-triol[4] has been isolated from normal urine (32, 33) and from the urine of patients with adrenal tumors (28, 30). As pointed out by Hirschmann (28) and Mason and Kepler (30), the structural similarity between dehydroisoandrosterone and Δ^5-androstene-3(β),16(β),17(α)-triol suggests that the former is the logical precursor of the latter. In addition, the conversion of a 17-ketone to a 16,17-glycol is of known natural occurrence, as attested by the formation of estriol from estrone in the human (34). The rôle of Δ^5-androstene-3(β),17(α)-diol as a possible intermediate in the conversion of dehydroisoandrosterone to androstenetriol may be decided in part by incubating the diol disuccinate with liver or other tissues.

The isolation of an α,β-unsaturated ketone in these experiments is of doubtful significance in view of the fact that a similar type of compound was formed in those control experiments in which liver slices alone were incubated. Although it appears that this compound may have been formed from the cholesterol present, proof must await further experimentation.

In order to rule out the factor of bacterial contamination and activity in the longer incubations, in Experiment 1 incubation was intentionally limited to a period of 3 rather than 6 hours. Since the products were qualitatively the same in both cases, it is apparent that this factor was not involved.

In view of the losses of steroid incurred in the course of the various manipulations, it is not possible, on the basis of these experiments alone, to estimate the extent to which metabolic processes converted the steroid to substances no longer recoverable as such.

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[4] The spatial configurations of the C-16 and C-17 hydroxyl groups have recently been defined by Huffman and Lott (31).

SUMMARY

1. Sodium dehydroisoandrosterone hemisuccinate was incubated with surviving rabbit liver slices under aerobic conditions for periods of 3 and 6 hours at 38–40°.

2. The products isolated were Δ^5-androstene-3(β),17(α)-diol, which was obtained in yields of from 43 to 69 per cent, Δ^5-androstene-3(β),16(β),17(α)-triol, which was isolated in yields of from 2.4 to 8.9 per cent, and an as yet unidentified α,β-unsaturated ketone, which was formed in yields of about 1 per cent. The formation of a second, unidentified α,β-unsaturated ketone in certain control experiments is described and its possible importance is discussed.

3. The results have been discussed in relation to the metabolism of dehydroisoandrosterone in general and in relation to pertinent *in vivo* studies previously reported.

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METABOLITES OF 11-DEHYDROCORTICOSTERONE: PREGNANE-3(α),20-DIOL-11-ONE

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A study[1] which involved the administration of relatively large amounts of synthetic 11-dehydrocorticosterone (Ia) and its acetate[2] (Ib) to a woman (Case 1) and to a man (Case 2) with Addison's disease afforded an opportunity to determine what urinary products might be related to this hormone. Thus far it has not been possible to isolate from the urine, in cases of hyperfunctioning lesions of the adrenal cortex, any 11-oxygenated pregnane derivatives. In the present study pregnane-3(α),20-diol-11-one and another unidentified compound were isolated from the urine collected while 11-dehydrocorticosterone and its acetate were being given. These compounds could not be found in the urine during another period when 17-hydroxy-11-dehydrocorticosterone was being given.

DISCUSSION

The urine was processed, the neutral extracts were separated into "ketonic" and "non-ketonic" fractions, and these fractions were subjected to chromatographic analysis essentially as previously described (1). The only crystalline fractions of any consequence were isolated from the non-ketonic fractions. Only a trace of crystalline material was obtained from the ketonic fraction. It was necessary to rechromatograph the crystalline material first obtained from the non-ketonic fraction in order to separate it into two compounds. The compound that was not identified had a melting point a little lower than that of pregnanediol, and its melting point was not depressed by admixture of pregnanediol. However, the acetate of the newly isolated compound melted at 147–149°, whereas a specimen of pregnanediol diacetate melted at 160–162°.

Analysis of the other compound indicated the formula $C_{21}H_{34}O_3$. When the compound was heated 1 hour at 90° with pyridine and acetic anhydride, a diacetate was formed. The formula, $C_{21}H_{34}O_3$, indicated the presence of a ketone group or of a carbon-carbon double bond. A double bond would require the presence of a third alcohol group in a hindered position, since it was not acetylated. If a ketone group were present, it also must be in a hindered position such as C-11, since it did not combine with Girard's reagent. (The ketonic fraction was chromatographed, but there was no evidence of this compound in that fraction.) Since it is known that 11-desoxycorticosterone can be converted physiologically to pregnanediol (2–4), it seemed likely that this new compound might be 11-ketopregnanediol (II) (pregnane-3(α),20-diol-11-one). Consequently, it was oxidized so that the ketone formed might be compared with pregnane-3,11,20-trione (III). This latter compound had been prepared by Dr. R. B. Turner in Dr. E. C. Kendall's laboratory; I am indebted to them for this preparation. The melting points of the two preparations were identical and there was no depression in the melting point of a mixture. Likewise there was no depression in the melting point of a mixture of the oximes.

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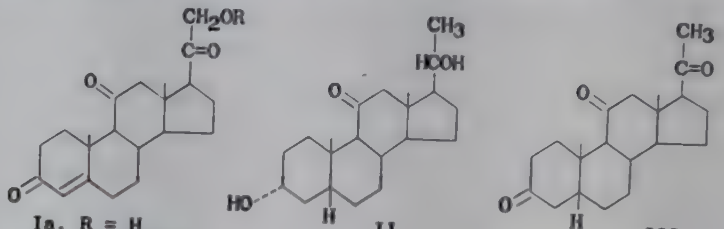
[1] Sprague, R. G., Gastineau, C. F., Mason, H. L., and Power, M. H., *Am. J. Med.*, in press.

[2] These substances were provided through the courtesy of Merck and Company, Inc., Rahway, New Jersey.



Ia. R = H
Ib. R = CH_3CO
II
III

Lieberman reported at the Laurentian Hormone Conference, September 10, 1947, the isolation of pregnane-3(α),20-diol-11-one from the urine of normal individuals. The excretion of pregnane-3(α),20-diol-11-one as the result of administration of 11-dehydrocorticosterone definitely identifies this latter hormone as the precursor of the urinary compound.

During the administration of 11-dehydrocorticosterone and its acetate there was no increase in the excretion of 17-ketosteroids as determined by the Zimmermann reaction. Furthermore, it was not possible to isolate any crystalline substance from the ketonic fraction. It may be concluded that 11-dehydrocorticosterone is not a precursor of 17-ketosteroids, but that it is converted in small part to pregnane-3(α),20-diol-11-one and in large part to unrecognized substances. Possibly this latter part is destroyed completely.

EXPERIMENTAL

In Case 1, 50 mg. of 11-dehydrocorticosterone acetate were given daily for 8 days and then 200 mg. for 11 days. The urine collected during administration of the acetate (Period 1) and during the 2 days following withdrawal of the hormone was pooled. Later, 100 mg. of the free hormone were given daily for 11 days (Period 2). The urine collected during this period was processed apart from that collected during Period 1. In Case 2, 100 mg. of 11-dehydrocorticosterone were given daily for 5 days.

After the addition of 50 ml. of chloroform, the 24 hour specimens of urine were stored in the refrigerator at 5° for 2 days. They were then extracted with chloroform for determination of "cortin-like" substances. The urine residues were acidified with 5 ml. of concentrated HCl and stored in the refrigerator until the collection was complete. The pool of urine was then concentrated and worked up in the manner that has been described previously (1).

In Case 1, 20 mg. of 17-hydroxy-11-dehydrocorticosterone also were given daily for 11 days. Nothing crystalline could be isolated from the urine collected during this period, perhaps because of the relatively small daily dose of hormone. However, since the results were completely negative, this period served as a control for the periods during which 11-dehydrocorticosterone was administered. Any steroids isolated during the latter periods must have been derived from 11-dehydrocorticosterone. The amount of steroids normally excreted by these patients, as measured by the Zimmermann reaction, was negligible.

The chloroform extracts obtained before acid hydrolysis also were examined. The ketonic portion of the fraction that was removed from benzene by repeated extraction with water was used for the estimation of cortin-like substances. Not enough remained for any attempt to isolate compounds. The non-ketonic fraction, however, yielded a crystalline substance which melted at 272° and which appeared to be too soluble in water to be of steroid nature. It was also present when 17-hydroxy-11-dehydrocorticosterone was given. It will not be considered further in this report, since it evidently bore no relation to 11-dehydrocorticosterone.

Ketonic Fraction—The ketonic fractions of the neutral extract of the urine that had been boiled with HCl gave only traces of a crystalline compound. This result was expected, since the excretion of 17-ketosteroids as determined by the Zimmermann reaction was 0.3 to 1.5 mg. per day and was not increased by administration of the hormone.

Non-Ketonic Fraction—The non-ketonic fractions obtained in Case 1 during administration of 11-dehydrocorticosterone acetate and during administration of the free hormone were 370 and 200 mg., respectively, and that obtained in Case 2, 145 mg. These three non-ketonic fractions were chromatographed separately on alumina. Benzene containing increasing amounts of alcohol was used for elution of the various fractions.

In Period 1, Case 1, a few crystals were obtained from the fraction eluted with 0.2 per cent of alcohol by volume. After recrystallization from acetone they melted at 200–210°. Not enough was available for further work. The fraction eluted with 1 per cent alcohol weighed 90 mg. Analysis of the recrystallized fraction (50 mg.) and its acetate indicated that it was not a single compound. Consequently the remainder was combined with the corresponding fraction obtained in Period 2 (total weight 80 mg.) and rechromatographed.

Compound with Melting Point of 232–233°—In the second chromatogram 11 mg. of a crystalline fraction were eluted with the fourth to ninth 20 ml. portions of 0.3 per cent alcohol by volume in benzene. The substance melted at 232–233°. A mixture with pregnane-3(α),20(α)-diol (m.p. 235–237°) melted at 231–233°. However, the acetate of this substance melted at 147–149° and therefore could not be the acetate of pregnanediol. A mixture with etiocholane-3(α),17(α)-diol (m.p. 233°) melted at 216–222°. There was not sufficient material for further characterization.

Pregnane-3(α),20-diol-11-one (II)—Beginning with the sixteenth 20 ml. portion of 0.3 per cent alcohol in benzene, 100 ml. of solvent removed 7 mg. of crystalline material; 100 ml. of 0.5 per cent and 100 ml. of 1 per cent alcohol in benzene together eluted 36 mg. of the same material. It was best recrystallized by addition of acetone to its solution in a little hot methanol. It melted at 217–219°; $[\alpha]_D^{27} = +59° \pm 2.2°$ ($c = 0.407$ in alcohol). It failed to give a precipitate with digitonin in 90 per cent alcohol. For analysis a sample was dried 1 hour at 100° and 0.1 mm.

Analysis—$C_{21}H_{34}O_3$. Calculated, C 75.33, H 10.14; found, C 75.40, H 10.25

Pregnane-3(α),20-diol-11-one Diacetate—The acetate was prepared by heating 10 mg. with a few drops of pyridine and 3 drops of acetic anhydride for 1 hour at 90°. Water was added while cooling; then sufficient concentrated HCl was added to make the mixture acid to Congo red. The precipitate was filtered out, washed with dilute HCl and water, dried, and recrystallized from methanol. The first crop was recrystallized, and it then weighed 7 mg. and melted at 233°.

Analysis—$C_{25}H_{38}O_5$. Calculated, C 72.08, H 8.98; found, C 71.74, H 9.15

Oxidation of Pregnane-3(α),20-diol-11-one—To a solution of 12 mg. of this compound (0.144 milliequivalent) in 1 ml. of glacial acetic acid was added 0.26 ml. of CrO_3 in acetic acid containing 0.158 milliequivalent (10 per cent excess). The mixture was allowed to stand overnight. Benzene was added and the solution was washed three times with water, then with sodium carbonate solution, and finally with water. Removal of the benzene and crystallization of the residue from anhydrous ether gave crystals which melted at 156–157°. A mixture with pregnane-3,11,20-trione (III) (m.p. 157–158°) melted at 156–157°.

The oximes also were prepared for comparison. The ketone (2 mg.), 0.5 ml. of a solution of hydroxylamine hydrochloride (4 mg. per ml.) in 50 per cent alcohol, and 2 drops of a saturated solution of sodium acetate were heated in an open tube in a water bath until the alcohol had evaporated. Heating was continued while alcohol was added dropwise until the precipitate had redissolved. The crystals that separated on cooling were washed with water and dried. The oxime prepared from the authentic specimen of pregnane-3,11,20-trione melted at 240–243° when placed on the block at 230°. The other oxime melted at 243–245°. A mixture of the two melted at 240–244°.

SUMMARY

Pregnane-3(α),20-diol-11-one was isolated from the urine of two patients with Addison's disease after administration of 11-dehydrocorticosterone and its acetate. Another substance melting at 232–233° was isolated but not identified.

Microanalyses were performed by J. Alicino. Melting points were determined with the Fisher-Johns electrically heated block and are recorded as read.

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16-SUBSTITUTED STEROIDS

V. THE PARTIAL SYNTHESIS OF URINARY ANDROSTENETRIOL (Δ^5-ANDROSTENE-3(β),16(β),17(α)-TRIOL)

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In 1943 Hirschmann (1) isolated from the urine of a boy with adrenocortical carcinoma a new triol in the steroid group. His skilful studies in characterization and degradation showed this new steroid to be a Δ^5-androstene-3(β),16,17-triol. This compound occurred in strikingly high amounts (95 mg. per liter, or more) in the urine studied by Hirschmann.

Marrian (2, 3) subsequently showed that Hirschmann's triol is a constituent of normal human urine (both male and female). Marrian had had this same triol in hand at a much earlier date, but had been unable to establish an empirical formula for it; however, a reexamination of his compound in the light of Hirschmann's research showed clearly the identity of the two substances. According to Marrian and Butler (3) urinary androstenetriol is present to the extent of only 0.1 mg. per liter in normal human urine.

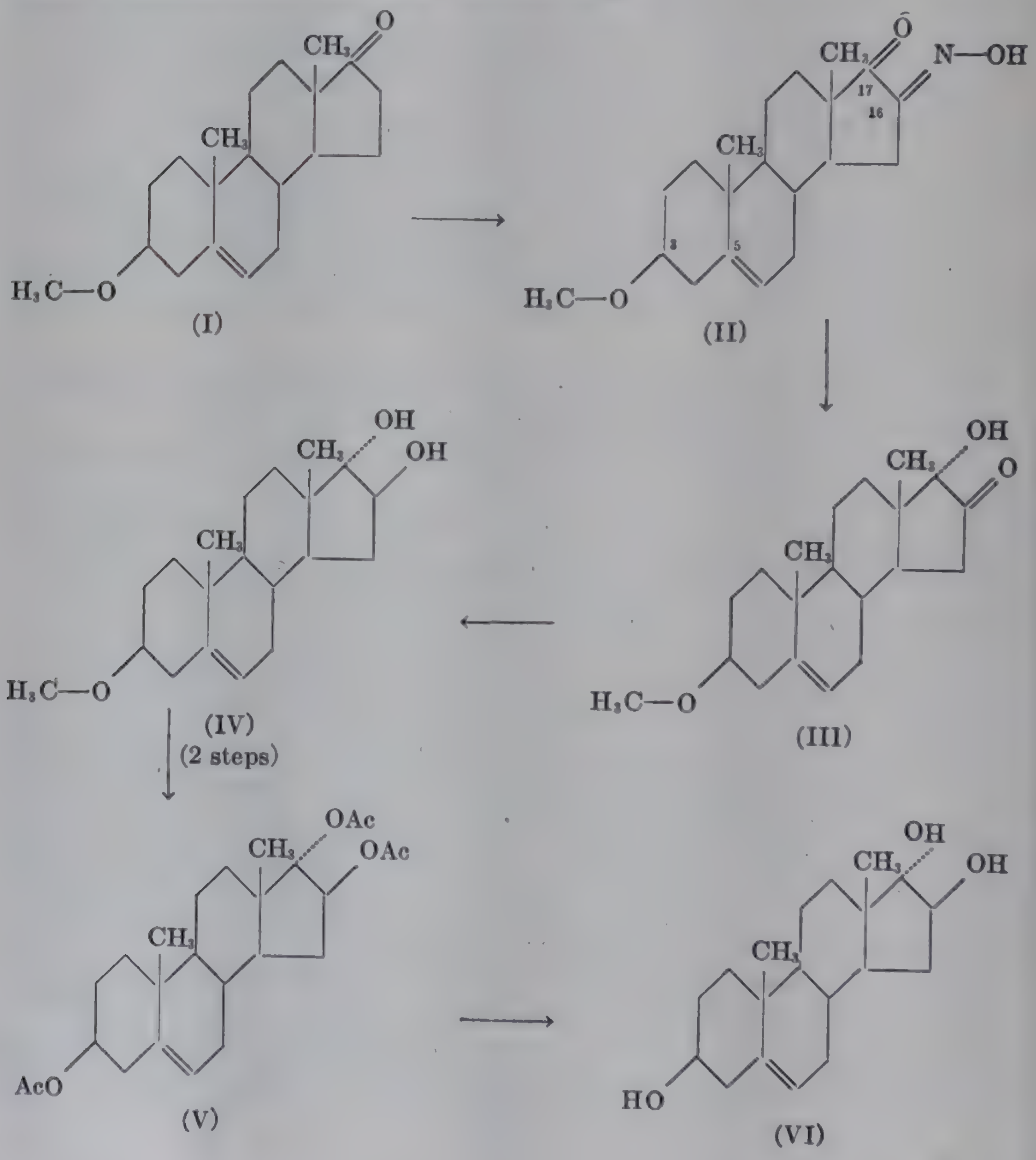
Mason and Kepler (4) have also reported the presence of Hirschmann's triol in the urine of a patient with an adrenal cortical tumor.

We recently announced (5) the preparation of urinary androstenetriol from dehydroisoandrosterone and the confirmation of Hirschmann's structural assignment for the molecule. Our method of synthesis, practically identical with the one used to prepare the sex hormone estriol (6, 7), allowed us to state that Hirschmann's triol and estriol possess the same spatial configuration of carbinols at C_{16} and C_{17} and that these carbinols are transoid in arrangement. This assertion was made possible from the following reasons: (*a*) the α-ketol 3-methyl ether obtained from the Stodola reduction of 16-oximinoestrone 3-methyl ether furnishes, upon further reduction with sodium amalgam in excess dilute ethanolic acetic acid (40°), estriol 3-methyl ether, which possesses the C_{16}–C_{17} hydroxyl groups in transoid arrangement, as the latter compound gives no cyclic acetonide (5); (*b*) the Stodola reduction of 16-oximinodehydroisoandrosterone 3-methyl ether yields an α-ketol which furnishes, upon further reduction with sodium amalgam as just described, the 3-methyl ether of Hirschmann's triol, a glycol which is likewise transoid at C_{16}–C_{17}; (*c*) since the two α-ketol 3-methyl ethers were prepared in completely analogous fashions, it was logical to suppose that they possess identical configurations at C_{16}–C_{17}

and that a common transoid form of the C_{16}–C_{17} glycol would arise from the same method of reduction.

Very recent research (8) now permits us to assign steric structures for all known C_{16}–C_{17} glycols in the steroid group. The subject of steric structure will be treated fully in Paper VI in this series; suffice it to say here that Hirschmann's triol is Δ^5-androstene-3(β),16(β),17(α)-triol and that estriol is $\Delta^{1,3,5}$-estratriene-3,16(β),17(α)-triol.

Ruzicka, Prelog, and Wieland (9) in 1945 surmised that estriol and urinary androstenetriol possess the same steric configuration at Ring D. These workers based their view on a clever comparison of differences in molecular rotation as measured between the naturally occurring triols and certain closely related steroid triols of synthetic origin.



In the present preparation of Hirschmann's triol, dehydroisoandrosterone 3-methyl ether was taken through the steps of nitrosation, Stodola reduction, and sodium amalgam reduction, as previously described (7), in the partial synthesis of estriol (I → II → III → IV). At this stage there was obtained the 3-methyl ether of urinary androstenetriol, which for a long time defied all attempts to split it to the free hydroxyl at C_3. It was eventually discovered that the methoxy group could be simply and smoothly substituted by an acetoxy group upon reaction with acetic anhydride in the presence of *p*-toluenesulfonic acid as a catalyst (IV → V). This reaction has proved a general one for the transformation of 3-methoxy-Δ^5-steroids to 3-acetoxy-Δ^5-steroids. The yields are high, and only a brief reaction time is required. The acetoxy group is, of course, readily saponified to give the free C_3-OH (V → VI).

From dehydroisoandrosterone 3-methyl ether, Hirschmann's triol (as triacetate and recovered free triol) was prepared in 21 per cent over-all yield. Dehydroisoandrosterone 3-methyl ether may be prepared from cholesteryl methyl ether or obtained by the methylation of dehydroisoandrosterone (10).

EXPERIMENTAL[1]

Dehydroisoandrosterone 3-Methyl Ether—This compound was prepared in a fashion similar to that of Butenandt and Grosse (10), whose method is based on Stoll's general procedure (11) for the formation of normal ethers of cholesterol. In our reactions, the *p*-toluenesulfonic acid ester was not purified but submitted, after drying, directly to reaction with methanol.

Nitrosation of Dehydroisoandrosterone 3-Methyl Ether—Metallic potassium (0.33 gm.) was dissolved in 25 cc. of dry tertiary butyl alcohol with mechanical stirring. The resulting solution of potassium tertiary butoxide was then poured onto 805 mg. of dehydroisoandrosterone 3-methyl ether (m.p. 137.5–138°) (I), and the mixture gently warmed until solution of the steroid was complete. The solution, cooled to near room temperature by mechanical stirring, was treated with 0.60 cc. of isoamyl nitrite. Stirring was continued for 6 hours, and the reaction mixture then left overnight at room temperature. The solution plus precipitated material was transferred to a separatory funnel by means of 400 cc. of aqueous glycine (≈7.0 gm. of glycine), and, following the addition of 7.0 cc. of concentrated hydrochloric acid, partitioned with 400 cc. of ethyl ether. The ethyl ether, after the aqueous acid phase had been separated off, was washed once with 300 cc. of 3 per cent sodium bicarbonate, and then extracted once with 200 cc. and again with 100 cc. of 0.5 N potassium hydroxide. Acidification of the combined potassium hydroxide phases with concentrated hydrochloric acid precipitated the 16-oximino derivative. This material was allowed to sett in the ice box and then filtered and washed copiously with water. Th yield of pale yellow crystals (oven-dried) was 603 mg., decomposing 171.5–173.5°. Of this material, 103 mg. were recrystallized twice fro aqueous methanol (charcoal) to give 92 mg. of tiny, near white crystals d composing at 180–183.5° (II).

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[1] All melting points are uncorrected.

Analysis—$C_{20}H_{29}O_3N$. Calculated. N 4.23
Found. (*a*) " 4.20
(*b*) " 4.14

Two Stage Reduction of 16-Oximinodehydroisoandrosterone 3-Methyl Ethe to 16,17-Glycol—To 500 mg. of 16-oximinodehydroisoandrosterone 3-methy ether (m.p. 171.5–173.5°, with decomposition) (II) were added 34 cc. of 5 per cent acetic acid and 1.4 gm. of zinc dust. The mixture was reflux for 1 hour and the hot solution decanted from the zinc into 175 cc. of wate The zinc was rinsed twice with 4 cc. portions of acetic acid and thes united with the main reaction solution in water which was subsequentl partitioned with 200 cc. of ethyl ether; the latter, after separation, wa washed with 0.5 N hydrochloric acid, with 1.0 N sodium hydroxide, an with water. Evaporation of the ethereal phase yielded a nice white crystalline product, Δ^5-androstene-3(β),17(α)-diol-16-one 3-methyl ether (III) It was not further purified but directly submitted to reduction with sodiu amalgam as given in the following paragraph.[2]

To the solution of the α-ketol (as above) in 58 cc. of ethanol and 6.5 cc. o acetic acid were gradually added 125 gm. of 2 per cent sodium amalgam the temperature being carefully maintained at 40.0–40.5° by continuous swirling in a water bath. (As soon as sodium acetate commenced t precipitate, 5.0 cc. of 50 per cent acetic acid were added.) When the reaction had been completed, the mixture of mercury and solution was diluted with water and extracted with ethyl ether. After removal of the mercury and aqueous phases the ether was further washed with water, with 0.5 N sodium hydroxide, and again with water. Evaporation of the ether yielded a white crystalline product, which after recrystallization, twice from acetone-Skellysolve B, once from absolute acetone, and twice from aqueous ethanol, melted at 196–197° (100 mg.). From aqueous ethanol, Δ^5-androstene-3(β),16(β),17(α)-triol 3-methyl ether crystallized as the hemihydrate in the form of tiny leaves (IV).

Analysis—$C_{20}H_{32}O_3 \cdot \frac{1}{2}H_2O$. Calculated. C 72.92, H 10.09
Found. (*a*) " 72.94, " 10.04
(*b*) " 72.93, " 9.95

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[2] As in the partial synthesis of estriol (7), it was finally decided that isolation of the intermediate α-ketol 3-methyl ether in pure form held no advantage.

From the extracted mother liquors were recovered by repeated crystalization from acetone-cyclohexane and from aqueous ethanol an additional 101 mg., melting at 195–196°.

Transformation of Cholesteryl Methyl Ether to Cholesteryl Acetate—Pure cholesteryl methyl ether[3] (0.50 gm.) and *p*-toluenesulfonic acid monohydrate (Eastman, No. 984) (0.25 gm.) were covered with 25 cc. of redistilled acetic anhydride and heated on the steam bath with frequent stirring for exactly 30 minutes. The solution was cooled at once under the tap and then placed in the ice box. After 24 hours the product was filtered, washed with 90 per cent acetic acid, and washed well with water. The fine crystals (dried *in vacuo*) weighed 0.37 gm. and melted at 113–113.5°. A mixed melting point test performed with authentic cholesteryl acetate showed no depression.

The mother liquor was apparently quite rich in cholesteryl acetate but no attempt was made to recover it.

Transformation of Urinary Androstenetriol 3-Methyl Ether to Triacetate—To 301 mg. of the 3-methyl ether of Hirschmann's triol (m.p. 195–196°) (IV) dissolved in 5 cc. of dry pyridine were added 3 cc. of acetic anhydride. The two phases were mixed well and left 24 hours with occasional swirling. The diacetate was then precipitated by treatment with 200 cc. of ice water, and finally, after 3 hours in the ice box, filtered and washed copiously with water.

To the air-dried diacetate (as above) were added 186 mg. of *p*-toluenesulfonic acid monohydrate and the solid materials covered with 18.6 cc. of redistilled acetic anhydride. The mixture was heated on the steam bath with frequent stirring for exactly 30 minutes and then cooled in an ice bath. As no crystallization of triacetate had occurred within 3 hours time, the anhydride was decomposed with ice water and the steroid extracted with ethyl ether. The ether, after having been washed with aqueous sodium bicarbonate and with water, was dried and then evaporated. The resulting white residue was three times recrystallized from aqueous ethanol (once with the aid of charcoal) to yield 249 mg. of white plates, m.p. 183–184°. Of this product, 100 mg. were recrystallized from 3.0 cc. of ethanol (ice box) to give 70 mg. of colorless plates melting at 184–185° (V).

Analysis—$C_{25}H_{36}O_6$. Calculated. C 69.42, H 8.39
Found. (*a*) " 69.36, " 8.41
(*b*) " 69.45, " 8.50

Dr. Hans Hirschmann performed a mixed melting point test with his urinary androstenetriol triacetate and our synthetic material and found no depression of the melting point.[4]

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[3] Prepared in high yield by the method of Stoll (11), m.p. 84°.

[4] Hirschmann, H., personal communication.

Δ^5-Androstene-3(β),16(β),17(α)-triol—All filtrates from the recrystallizations of triol triacetate in the preceding section were added to ether and the ethereal solution washed several times with water. After evaporation of this ether, the resulting crystalline residue was dissolved in 25 cc. of ethanol, 25 cc. of 1.0 N potassium hydroxide were then added, and the alkaline solution refluxed for 30 minutes. Following the addition of 25 cc. of water the saponification medium was distilled until it had become turbid. The free triol was allowed to crystallize for 2 days in the ice box and then filtered and washed well with water (100 mg., melting at 250–252.5°). This material was recrystallized once from aqueous ethanol, once from absolute acetone, and again from aqueous ethanol (charcoal), whereupon it weighed 70 mg. and melted at 257.5–260.5° with some decomposition (VI). From aqueous ethanol the triol crystallized as hexagonal plates with one-half molecule of water.

Analysis—$C_{19}H_{30}O_3 \cdot \frac{1}{2}H_2O$. Calculated. C 72.34, H 9.91
Found. " 72.37, " 9.97

Dr. Hirschmann found no depression of melting point when his urinary androstenetriol was melted with our synthetic triol.[4]

Acetylation of Δ^5-Androstene-3(β),17(α)-diol-16-one 3-Methyl Ether—Crude α-ketol (0.51 gm.) (III), direct from Stodola reduction without purification, was acetylated with acetic anhydride (4 cc.) in pyridine (6 cc.) as usual. The crude acetate, obtained by precipitation with ice water, was crystallized from aqueous ethanol (charcoal) to give 0.56 gm. of flat plates (m.p. 147.5–150°). Two recrystallizations from absolute methanol yielded 0.29 gm. of material melting at 165–168°. A third recrystallization from methanol raised the melting point to 166–169° (0.25 gm. of flat needles).

Analysis—$C_{22}H_{32}O_4$. Calculated. C 73.30, H 8.95
Found. (*a*) " 73.30, " 9.07
(*b*) " 73.12, " 8.99

This material is structurally Δ^5-androstene-3(β),17(α)-diol-16-one-17-acetate 3-methyl ether (8).

SUMMARY

The partial synthesis of urinary androstenetriol (Hirschmann's triol) is described in detail. From dehydroisoandrosterone 3-methyl ether it is possible to prepare the substance in 21 per cent over-all yield.

A new general method for the transformation of 3-methoxy-Δ^5-steroids to 3-acetoxy-Δ^5-steroids is described.

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STUDIES ON THE STRUCTURE OF POLYSACCHARIDES

I. ACID HYDROLYSIS OF STARCH-LIKE POLYSACCHARIDES*

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(Received for publication, July 28, 1947)

During the course of an investigation of the activating effect of polysaccharides and their split-products on phosphorylases, it became necessary to study the kinetics of acid hydrolysis of polysaccharides. The following cases were analyzed: linear polymers containing α-1,4 linkages (amylose), branched polysaccharides containing both α-1,4 and α-1,6 linkages (amylopectin, glycogen), polymers containing α-1,6 linkages (bacterial dextran), and cyclic structures containing α-1,4 linkages (the Schardinger dextrins).

The question of the relative stabilities of the α-1,4 and α-1,6 linkages as found in amylopectin and glycogen has been a matter of disagreement. Data have been published supporting the opinion that the 1,6 is the less stable linkage (1, 2), that it is the more stable (3, 4), and that the two are hydrolyzed at the same rate (5). We have found that at least under conditions of high acidity and low temperature the α-1,4 is definitely more easily hydrolyzed than the α-1,6 linkage.

The hydrolysis of the Schardinger dextrins represents a special case. The opening of the rings by the splitting of one of the 1,4 linkages results in the formation of an open chain of 6 glucose units in the case of the α-Schardinger and of 7 glucose units in the case of the β-Schardinger dextrin. These materials are of particular advantage in an investigation of the structural characteristics necessary for the activation of phosphorylase.

EXPERIMENTAL

Methods

The conditions of the hydrolysis were similar to those of Hidy and Day (6), except that the temperature was maintained at 30° in a thermostatically controlled water bath. The sample was weighed out into a volumetric flask, wetted with water, and made to volume with concentrated HCl, while

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the flask was cooled in water. The concentration of the polysaccharide was 0.1 per cent. Titration of the chloride by the Volhard method showed that the hydrochloric acid concentration varied between 7.5 and 7.7 N in different series of experiments. Samples were removed at suitable intervals, neutralized with KOH, and diluted to definite volume.

It was possible to calculate the degree of hydrolysis from measurements of reducing power made with an alkaline copper reagent, in spite of the fact that such reagents have hitherto been considered unreliable for estimating the longer saccharide chains. In preliminary experiments several reagents of the Somogyi-Shaffer type were tested. In order to evaluate their reliability as indicators of the degree of hydrolysis, the first order rate constant for amylose was compared to that for maltose. It was to be expected that a suitable reagent would give the same rate constant for the splitting of the maltosidic linkages in the linear polymer amylose as in the disaccharide maltose, provided that the reducing power was inversely proportional to the length of the chain. Weakly alkaline solutions such as the bicarbonate-carbonate Reagent 50 (7) did not fulfil the conditions. However, when Reagent 60 (7), containing only sodium carbonate as the alkali, was used with a boiling period of 30 minutes, it was found that the hydrolysis of amylose followed a first order course over a wide range of hydrolysis, and that the rate constant was the same as that found for maltose.[1] An example of the precision obtained in these measurements is given in Table I. These results are typical of those found in several similar experiments.

The rate constant was calculated according to the equation, $K = 1/t \log_{10} c/(c - x)$, where c = glucose found on complete hydrolysis, x = the reducing power in terms of glucose expressed as per cent of c, and t = time in minutes. The constants found for the first intervals measured were regularly too low, which shows that the reagent used was not sensitive enough to measure the true reducing power of the longest chains. In calculating the averages, the first and last values corresponding to about 5 and 95 per cent hydrolysis were arbitrarily omitted in all cases, since these are likely to be the least accurate in a series of this kind. The rate of hydrolysis of amylose usually remained constant until the very end of the reaction, while that of amylopectin and glycogen declined slightly (*cf.* Table I).

RESULTS AND DISCUSSION

The constants found for several polysaccharides are summarized in Table II and are given with the standard deviation of each series. The grand average for all the results of amylose, amylopectin, and maltose is

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[1] Maltose with this copper reagent had exactly one-half the reducing power of an equivalent quantity of glucose.

TABLE I

Hydrolysis Constants in 7.7 N HCl at 30°

The concentration of polysaccharides was 0.1 per cent.

Time	Amylose		Amylopectin	
	Hydrolysis	$K \times 10^3$	Hydrolysis	$K \times 10^3$
min.	*per cent*		*per cent*	
30	5.4	0.77*	6.3	0.95*
60	13.7	1.07	15.0	1.07
120	25.0	1.04	26.0	1.08
180	35.4	1.06	37.6	1.14
240	44.0	1.05	46.3	1.10
300	54.0	1.12		
360	57.8	1.04	58.7	1.07
420			63.4	1.03
480	69.0	1.06	70.0	1.09
600	76.0	1.00	77.5	1.08
1380			95.4	0.97*
1440	96.5	1.03*		
		1.05 ± 0.02		1.08 ± 0.03

* Not included in the average.

TABLE II

Hydrolysis Constants of Various Polysaccharides in 7.7 N HCl at 30°

The concentration of polysaccharides was 0.1 per cent.

Polysaccharides	Source	$K \times 10^3$	No. of determinations
Maltose	Commercial	1.04 ± 0.06	6
Amylose	Schoch	1.05 ± 0.02	8
		1.06 ± 0.05	11
		1.06 ± 0.02	9
Amylopectin	"	1.07 ± 0.01	9
		1.08 ± 0.03	8
Limit dextrin from amylopectin*		0.95 ± 0.05	7
		0.96 ± 0.07	8
Glycogen	Rabbit liver	0.92 ± 0.015	6
		0.86 ± 0.04	7
Limit dextrin from glycogen*		0.89 ± 0.032	5
		0.89 ± 0.036	6
Dextran	Hehre	0.159 ± 0.004	3
		0.154 ± 0.004	7
		0.127 ± 0.005	7
	Hassid	0.119 ± 0.005	5

* Prepared by prolonged action of β-amylase on corresponding polysaccharides.

$K = 1.06 \times 10^{-3}$. This is also the slope of the straight line obtained by plotting the function $\log_{10} (c - x)$ *versus* time for these materials.

From the fact that maltose and amylose are hydrolyzed at the same rate it can be concluded that the α-1,4 linkage between any 2 glucose units has a characteristic stability which is not dependent on the length of the chain in which it is situated. If no other factor than this characteristic stability influenced the rate of reaction, then the hydrolysis should be completely random and should be found to be kinetically of the first order. Since the reaction was actually found to be of first order, we can conclude that all the linkages in amylose are equally available to acid hydrolysis, at least under the conditions of our experiment.

Although the branched polysaccharide amylopectin has a much more complicated structure than amylose, it is hydrolyzed at the same rate and the reaction is of the first order; therefore all the 1,4 linkages are equally susceptible to the action of the acid. The similarity between the rates of splitting of amylose and amylopectin indicates either that the 1,6 bond at the branch point is split at the same rate as the 1,4 bond or that, if hydrolyzed at a different rate, the 1,6 bonds are too few to make a marked difference.

The somewhat lower rates of hydrolysis shown in Table II for glycogen and the limit dextrins of glycogen and amylopectin, materials which contain a larger proportion of 1,6 linkages than amylopectin, suggest that the 1,6 bond is more difficult to hydrolyze than the 1,4 bond.

This suggestion could be directly tested by the use of bacterially formed dextran, which has been shown by several workers to contain mainly α-1,6 linkages (8–10). As early as 1881 Bechamp (11) noted that this material was more difficult than starch to hydrolyze in acid and this has remained common knowledge, but has not been made use of in an analysis of the structure of starch and glycogen.

Two samples of dextran were tested. One, obtained from Dr. Hehre, was synthesized by enzymes from *Leuconostoc mesenteroides*. The other, obtained from Dr. Hassid, was part of a sample, the analysis of which has already been reported (10). The rate of hydrolysis of dextran, about 12 per cent of that of amylose (Table II), is of the right order of magnitude to account for the results obtained with glycogen. As calculated from the respective velocity constants (1.06 and 0.14×10^{-3}), 97.0 per cent of the 1,4 and 37 per cent of the 1,6 linkage would be hydrolyzed in 24 hours. If glycogen contains 91 per cent of 1,4 bonds, then 0.97×91 or 88.3 per cent of the total hydrolysis is accounted for by the 1,4 linkages. In addition, 0.37×9 or 3.3 per cent is contributed by the 1,6 bonds, or glycogen should be 91.6 per cent hydrolyzed in 24 hours. Several determinations average 91 per cent hydrolysis in this time. Compared to this, amylose is found to be 97 per cent hydrolyzed in the same period. The assumption that the

1,6 linkage is split at the same rate in glycogen as in dextran is apparently supported by these calculations.

The cyclic Schardinger dextrins do not show the same regularity of hydrolysis as the other polysaccharides (12). These compounds have been shown to be rings of glucose units all joined through 1,4 linkages (13), the α-dextrin containing 6 units per molecule, the β-dextrin seven (14).

In Table III it is shown that K calculated from reducing power is not constant, but increases with time. This might be interpreted in terms of a consecutive reaction; that is, the ordinary random hydrolysis of the 1,4 linkages would not occur until after the ring has been opened. In this case one would be dealing with two rate constants, one representing the opening of the rings, the other the splitting of the open chain dextrins. The latter should correspond to that found from maltose and is, in fact, approached as the hydrolysis progresses (see Table III). In order to determine the rate constant for the opening of the rings, the open chain material present after different periods of hydrolysis was removed by treating it with β-amylase and destroying the resulting maltose by heating to 100° in N KOH for 90 minutes. The rings were then completely hydrolyzed in acid and determined as glucose. Control experiments showed that no Schardinger dextrin was lost by the β-amylase-alkali treatment, while 97 to 98 per cent of maltose or of partially hydrolyzed amylose (about 30 per cent hydrolysis in acid) was destroyed under these conditions.

TABLE III

Hydrolysis Constants of Schardinger Dextrins in 7.7 N HCl at 30°

Time	α-Dextrin				β-Dextrin			
	Reducing power		Ring opening		Reducing power		Ring opening	
	Hydrolysis	$K \times 10^3$	No. of rings broken	$K \times 10^3$	Hydrolysis	$K \times 10^3$	No. of rings broken	$K \times 10^3$
min.	*per cent*		*per cent*		*per cent*		*per cent*	
30	2.7	0.39	7.2	1.08*	3.2	0.47	15.7	2.46
60	7.5	0.57	18.7	1.49	8.0	0.60	29.0	2.48
120	16.3	0.65	34.6	1.54	18.5	0.74	50.1	2.50
180	25.8	0.72	48.0	1.58	27.6	0.78	66.6	2.65
240	31.7	0.69	58.7	1.60	36.8	0.83	78.3	2.74
360	46.0	0.74	75.1	1.67	51.2	0.87	88.7	2.63
540	67.0	0.89	89.2	1.78	67.8	0.91	95.7	2.54
660	77.1	0.97						
				1.61				2.57

* Not included in the average.

The splitting of *one* bond of the rings (K_1) was found to follow first order kinetics, the rate constant being 1.61×10^{-3} for the α-dextrin and 2.57×10^{-3} for the β-dextrin (Table III),[2] as compared to a rate constant (K_2) of 1.06×10^{-3} for the hydrolysis of open chains such as maltose and amylose. Since K_1 is greater than K_2, conditions are favorable for the accumulation of dextrins of short and fairly uniform chain lengths, and, as will be shown in Paper III of this series, these dextrins are able to activate potato phosphorylase.

It should not be concluded, however, that, because K_1 is greater than K_2, the bonds in the cyclic structure are less stable to acid than the same kind of a bond in an open chain. The two constants are not directly comparable for the following reasons: K_1 above was calculated from the concentration of unopened rings present at different times during acid hydrolysis and in this case only 1 out of 6 or 7 bonds is being considered, while all the bonds are being considered when rate constants are calculated from measurements of reducing power. Hence, it is necessary to approach the problem of stability of the bonds in a different manner.

One way of doing this is to calculate the probability that a ring will be opened when a 1,4 bond is split. Assume that there are 210 bonds present (*a*) in an open chain, (*b*) in the α-dextrin (thirty-five rings), and (*c*) in the β-dextrin (thirty rings), and that all the bonds have the same stability. Then $A = A(S)$, where A represents the most probable number of rings broken, when (S) bonds are broken.

$$A = \frac{N}{M}\left(1 - \left(1 - \frac{S-1}{N}\right)^M\right) + \left(1 - \frac{S-1}{N}\right)^M$$

where N represents the total number of bonds and M the number of bonds per ring.[3]

This equation is valid for $N >> S >> 1$. If $S = 21$, that is if 10 per cent of the bonds are broken, $A = 16.3$ for the α-dextrin and 15.6 for the β-dextrin, corresponding to a probability of 46.6 and 52 per cent of opened rings, respectively. The times at which 10, 20, etc., per cent bonds are broken are given by the known rate of hydrolysis of amylose, and this permits the calculated values to be plotted as rate curves. This has been done in Fig. 1, which also includes the actually observed rates for the opening of the rings of the α- and β-dextrin. The ratio of the predicted to the observed rates was 4.2 for the α-dextrin and 3.6 for the β-dextrin.

[2] These constants are somewhat lower than those previously reported (12). The earlier method involved removal of maltose by fermentation with yeast, and removal of the last traces of yeast by passing through a Seitz filter before acid hydrolysis. It was found that the cellulose filter pad retained significant amounts of the ring dextrins, presumably by absorption.

[3] The authors are indebted to Dr. Wiame for suggesting a statistical approach to this problem and to Dr. H. Primakoff for the development of this mathematical treatment.

According to this mathematical analysis the cyclic structures are much more stable to acid hydrolysis than would be predicted on the basis of the known rate of hydrolysis of the open chains.[4]

Experimental support for this conclusion was found in comparing the rates of acid hydrolysis at different temperatures. Between 30–40° the temperature coefficients were found to be 2.4 for amylose and 3.6 for β-Schardinger dextrin. This corresponds to E values calculated by the Arrhenius equation of 16,600 and 24,500 calories, respectively. The higher activation energy for the hydrolysis of Schardinger dextrins is in harmony with the idea that the ring forms are considerably more stable to acid than the open chains.

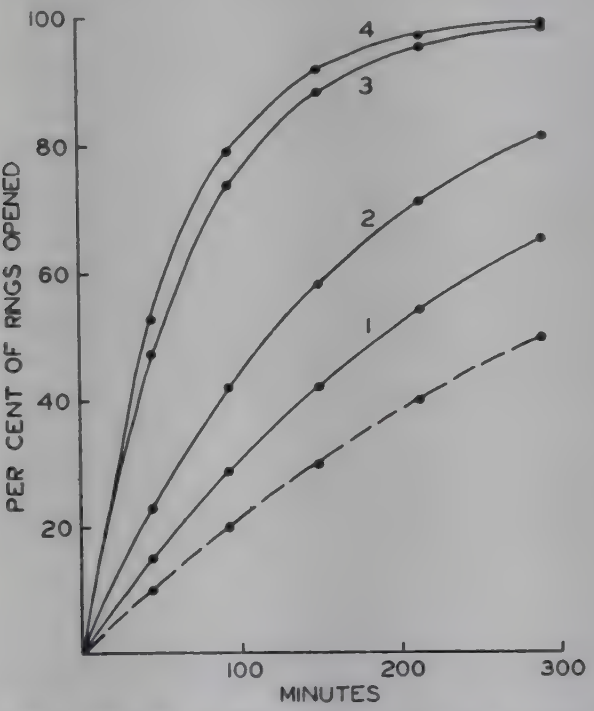

FIG. 1. Rate of ring opening of Schardinger dextrins during acid hydrolysis. Curves 1 and 2 experimentally determined values for α- and β-dextrins, respectively. Curves 3 and 4 predicted values, if the stability of the 1, 4 bonds was the same in the cyclic structures as in the open chains. For the equation used in calculating the values of Curves 3 and 4 see the text. The dotted line represents the rate of hydrolysis of amylose and fixes times at which 10, 20, etc., per cent of bonds are broken.

SUMMARY

1. Dilute solutions of various polysaccharides were hydrolyzed in 7.7 N HCl at 30° and the reducing power was determined by means of a copper

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[4] Some evidence has been obtained by Dr. Wiame in this laboratory that other cyclic structures, the metaphosphates, show similar characteristics of acid hydrolysis as the Schardinger dextrins.

reagent of high alkalinity. Closely agreeing first order constants were obtained for amylose, amylopectin, and maltose over a wide range of hydrolysis, showing that the α-1,4 linkages have the same stability in linear as in branched polysaccharides. Glycogen and the limit dextrin prepared from it and from amylopectin by the action of β-amylase were hydrolyzed at a measurably slower rate, particularly towards the end of hydrolysis. This was shown to be due to the relatively large proportion of α-1,6 linkages which occurs at the branch points. That this linkage is more resistant to acid hydrolysis than the 1,4 linkage was inferred from the fact that bacterial dextran, a polysaccharide containing mainly α-1,6 linkages, was hydrolyzed at a rate only 12 per cent that of amylose.

2. The kinetics of acid hydrolysis of the cyclic Schardinger dextrins were found to be complex. Based on the measurement of reducing power, a constantly increasing rate of hydrolysis was observed which approached that of maltose and amylose as hydrolysis neared completion. It was shown that there are two consecutive reactions involved, the first being the splitting of one bond of the rings which follows first order kinetics and occurs faster than the second reaction, the hydrolysis of the open chains.

3. An analysis of the data was presented which shows that the α-1,4 bonds in the ring structure are several times more stable to acid hydrolysis than the same bonds in open chains. Data on the energy of activation are in harmony with this conclusion.

The authors are indebted to Dr. Schoch for the supply of the starch fractions, to Dr. Hudson for the Schardinger dextrins, and to Dr. Hassid and Dr. Hehre for the bacterial dextran.

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STUDIES ON THE STRUCTURE OF POLYSACCHARIDES

II. DEGRADATION OF POLYSACCHARIDES BY ENZYMES*

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Starch and related polysaccharides are degraded by two different types of enzymes, the amylases and phosphorylases. An investigation of some of the properties of the phosphorylases involved the use of polysaccharides hydrolyzed by α- and β-amylase and led to a number of observations about the action of these enzymes which are recorded in this paper. It appears from these experiments that the action of α-amylase on amylopectin and glycogen is less random than its action on amylose, that β-amylase degrades one polysaccharide chain completely before transferring its activity to another, and that potato and muscle phosphorylases differ in their action on branch points.

α-Amylases—It is known that α-amylases from different sources differ in the details of their action on starch (1). Therefore all interpretations are meant to apply only to the enzyme actually used.

The α-amylases of both plants and animals are characterized by their dextrinogenic action. Starch is rapidly degraded to fragments showing progressively reddening iodine colors until the color reaction entirely disappears. Reducing dextrins, maltose, and small amounts of glucose account for the reducing power at each stage. No high molecular weight residue remains when the reaction has reached apparent completion. It has been suggested that the primary action may be the splitting off of fragments about 6 units long, with subsequent formation of maltose from these small dextrins (2). It seems that the 1,6 bonds at the point of branching cannot be hydrolyzed by the enzyme. Suggestive evidence on this point is the finding of a small amount of an apparently resistant residue from starch treated with α-amylase. The exact nature of this material has not been ascertained, but it is presumed to consist of tri- or tetrasaccharides

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containing a 1,6 linkage (3). In most of these investigations either native or some variety of soluble starch has been used.

In this work salivary amylase from one person was used throughout, and the substrates were liver glycogen and purified fractions of corn-starch. The saliva was collected fresh on the day used, diluted with water, and centrifuged to remove mucus. Repeated tests showed that it was of consistent amylolytic power. A slight trace of maltase activity was detectable only on long incubation (over 24 hours).

In order to test the ability of α-amylase to split 1,6 linkages, dextran was used as the substrate. To 100 mg. per cent solutions of dextran and starch respectively were added equal amounts of enzyme. In less than 2 minutes the starch was completely achromic to iodine and gave a positive reduction test with Benedict's solution. Even after 24 hours incubation at 30° the dextran showed no sign of reducing power. However, this negative result does not necessarily show that the enyzme is incapable of attacking a 1,6 linkage as it occurs in amylopectin, since the whole structure of the dextran may be such as to inhibit the enzyme. For example, there is no doubt that α-amylase can hydrolyze 1,4 linkages, but those in the Schardinger dextrins are completely resistant.

If the 1,6 linkages are not split, then amylopectin and glycogen should leave an unhydrolyzed residue of significantly greater chain length than that left by amylose. Samples of these three polysaccharides were dissolved in 3 N KOH and neutralized with HCl; then 20 ml. of 0.1 M citrate, pH 6.5, 20 ml. of 2 per cent NaCl, and water to make 100 ml. were added. These solutions were incubated with α-amylase for 40 hours, and then reducing power was determined before and after acid hydrolysis.[1] The average chain length in glucose units was found to be 2.0 for amylose, 3.7 for amylopectin, and 2.9 for glycogen. Since the formation of a small amount of glucose has not been excluded, the apparently complete conversion of amylose to maltose may be fortuitous, but, with this as a standard of comparison, it may be seen that the residues from the branched polysaccharides are significantly longer. This suggests that α-amylase does not split the linkage at the branch point.

An attempt was made to obtain evidence for the removal of fragments of uniform size. Polysaccharides were incubated with α-amylase at 30°, and samples were taken at intervals. The enzyme action was stopped by heat; then 1 aliquot of each sample was used for a determination of reducing power in order to calculate the per cent hydrolysis, while another aliquot was dialyzed 20 hours against 4 volumes of distilled water in the refrigerator. Dialysis was carried out in cellophane sacs obtained from the Visking Corporation. The reducing power of the inner and outer fluids was determined both before and after acid hydrolysis in order to calculate the chain length. From Tables I and II it can be seen that the dialyzable

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[1] The average chain length was calculated from the differences in reducing power before and after acid hydrolysis. The copper reagent used (see Paper I of this series) showed a reducing power per mg. of maltose hydrate which was one-half that of glucose.

TABLE I

Separation of Products of Hydrolysis of Glycogen Digested by α-Amylase

The samples were dialyzed in cellophane sacs for 20 hours against 4 volumes of H_2O at 2°.

Sample No.	Hydrolysis	Apparent chain lengths	
		Dialyzed solution	Dialysate
	per cent	*glucose units*	*glucose units*
1	1.6	87	4.0
2	2.5	72	2.4
3	5.8		4.7
4	7.1	24.4	3.7
5	14.7	16.3	3.3
6	21.2	12.5	3.6
7	23.7	9.2	3.7
8	25.0	8.4	3.6
9	26.2	8.0	3.7
10	28.8	7.0	

TABLE II

Separation of Products of Hydrolysis of Amylose Digested by α-Amylase

The samples were dialyzed in cellophane sacs for 20 hours against 4 volumes of H_2O at 2°. B. = blue, P. = purple, R. = red.

Sample No.	Hydrolysis	Dialyzed solution		Dialysate	
		Chain length	Iodine color	Chain length	Iodine color
	per cent	*glucose units*		*glucose units*	
1	3.2	21.2	B.-P.	8.6	Faint P.
2	7.8	17.7	P.	6.2	R.
3	10.3	15.3	“	5.7	“
4	12.2	10.5	R.-P.	5.6	“
5	13.5	9.8	“	4.5	“
6	16.2	8.3	R.	4.4	“
7	17.2	7.3	“	4.0	Faint R.
8	23.2	6.2	“	3.4	
9	28.8	4.3		2.9	
10	32.8	3.4		2.7	
11	40.5	2.7		2.6	

material obtained from glycogen was remarkably uniform in size over a fairly wide range of hydrolysis, while the dialyzable fragments from amylose showed no such uniformity. Furthermore, the undialyzable fragments of amylose showed a much greater reduction in size for a given degree of hydrolysis than those of glycogen. These results might be ascribed to differences in structure. The linear polymer, having a uniform and easily accessible structure, is hydrolyzed by α-amylase in a more random manner than the branched polysaccharide.

β-Amylase—This enzyme, found only in plants, has attracted much attention because of the peculiarities of its action. It is characterized as saccharogenic, since large amounts of reducing sugar and no detectable intermediates are formed. The generally accepted view of its action is that postulated by Hanes (4), which is that the enzyme successively removes maltose units from the non-aldehyde end of the chain until the action is blocked by some anomaly in structure. This block is now thought to be the 1,6 linkage at the branch point, since the unbranched fraction of starch is completely broken down.

Meyer and Bernfeld's (5) kinetic prediction that the rate of hydrolysis should remain constant because the number of end-groups remains the same implies that all the chains are shortened at a uniform rate. If this were true, then short chain intermediates should be obtainable from amylose partly digested by β-amylase.

Our β-amylase was prepared from ungerminated wheat by the method of Ballou and Luck (6). It showed no trace of maltase activity. Although the optimum pH is 4.8, it was very active for long periods of time at pH 6.5, which was the pH used for all but the preliminary experiments. A sample of amylose was dissolved in about 1 ml. of 3 N KOH and neutralized with HCl; then 20 ml. of 0.1 M citrate at pH 6.5 were added and enough water to make 100 ml. When the amylose concentration was below 200 mg. per cent, this amount of salt was sufficient to keep it from retrograding before complete enzymatic hydrolysis was achieved. About 0.5 mg. of enzyme powder in 1 ml. of citrate buffer was added, the digest incubated at 30°, and a sample taken at intervals. As with the α-amylase, the enzyme action was halted by heating at 100° for 10 minutes. 1 portion of each sample was used to determine the reducing power, which was calculated as maltose. Another portion was dialyzed in cellophane sacs against 50 volumes of water for 20 hours at 3°; as shown in Table II, fragments of an average chain length of 9 glucose units can be removed by dialysis. The material which was recovered from inside the sacs had no reducing power and stained blue with iodine; it was completely hydrolyzed in acid and determined as glucose.

The aim of this experiment was to calculate the amount of unhydrolyzed amylose (*a*) from the amount of undialyzable carbohydrate and (*b*) from the amount of maltose formed, the argument being that the formation of intermediate dextrins during the action of β-amylase should give rise to a discrepancy between these two values. The results are shown in Fig. 1, where the values for (*a*) and (*b*) are plotted as the per cent of the total amylose originally present. The two curves coincide up to a point where only 30 per cent of amylose is left unhydrolyzed; hence fragments other than maltose were apparently not formed in significant amounts. The divergence of the curves after more complete hydrolysis is due to the formation of a small amount of glucose, as is shown by the separate determination of maltose and glucose by differential fermentation with yeast. This makes the determination of the reducing power calculated as maltose too high and hence the hydrolysis of amylose appears more complete than it actually is. Had intermediate dextrins been present, the reducing power would have been determined too low, and the two curves would have diverged in the opposite direction.

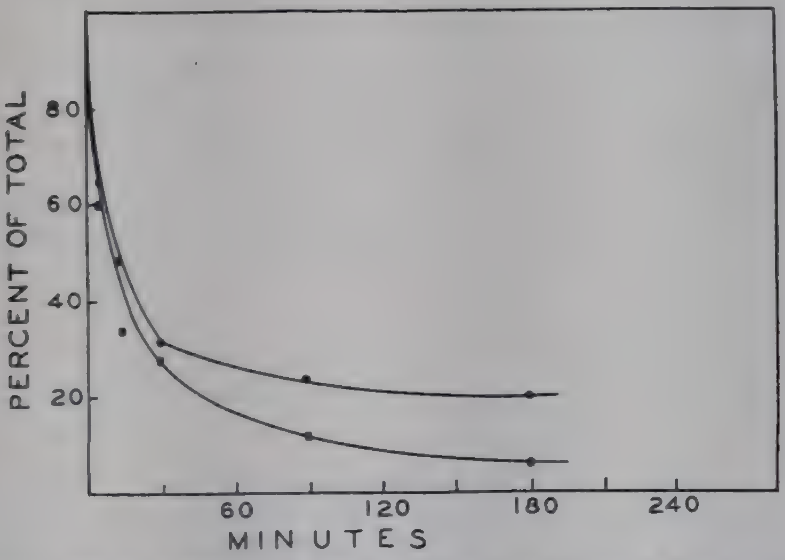

Fig. 1. Comparison of amount of amylose left unhydrolyzed after various intervals of digestion by β-amylase; ●, calculated from undialyzable carbohydrate; ■, calculated from determinations of reducing power as maltose.

The lack of short chain intermediates between amylose and maltose, the evidence from phosphorylase activation (Paper III) and from iodine colors (Paper IV), suggests that the action of β-amylase, at least on amylose, consists in complete scission of one whole chain to maltose before another chain is attacked. The same apparently applies to the action of β-amylase on amylopectin, since tests on the activation of phosphorylase show a decrease in the number of end-groups from the beginning of the reaction (Paper III). The action here is apparently the removal of one whole terminal chain as maltose before another is attacked, and not a shortening of all the outside chains at the same rate.

The question is sometimes raised whether or not the amylases can attack very short chains. When partial hydrolysates of α-Schardinger dextrin were treated with either α- or β-amylase, there was a marked rise in the reducing power of the solution, and practically all of the reducing power could be removed by fermentation with fresh yeast. Since the unopened rings were not attacked at all, this shows that both enzymes are able to split very short chains. A very small amount of non-fermentable reducing material which remained has not been investigated further.

Degradation by Phosphorylases—In an early study of mammalian phosphorylases Cori, Colowick, and Cori (7) showed that 95 per cent of the glycogen which disappeared could be accounted for as glucose-1-phosphate. It was also noted that, if an excess of phosphate was added, all the glycogen was degraded.[2] These findings suggest a resemblance to the end-wise attack of β-amylase, with the rather surprising additional ability to by-pass the 1,6 linkages at the branch points, or perhaps even to break them. The action of crystalline muscle phosphorylase on purified glycogen and amylopectin was studied, and the results which had been obtained with crude muscle extracts were confirmed.

The solution of polysaccharide (250 mg.) and enzyme was placed in a small dialyzing sac, suspended in a large volume of 0.1 M phosphate-cysteine buffer, pH 7.2, and incubated at 30°. A drop of toluene was added to the contents of the sac to inhibit bacterial growth. At intervals the outer solution was renewed so that glucose-1-phosphate was continually removed from the system. A control with citrate-cysteine buffer instead of phosphate was run simultaneously to check on the possible presence of amylases, and to correct for the effects of osmosis which tended to increase the concentration of glycogen. Samples were removed at intervals from both digests, the glycogen (or amylopectin) twice precipitated with alcohol, hydrolyzed in acid, and determined as glucose. The results are shown in Fig. 2, where the amount of polysaccharide remaining is plotted as per cent of that found in the control.

In order to find possible intermediates too small to be precipitated in alcohol, duplicate samples were hydrolyzed directly without precipitation. Identical amounts of glucose were found in both precipitated and non-precipitated samples. Even at the level of 80 per cent degradation the residue showed all the properties of the original glycogen; it was non-dialyzable, stained red with iodine, formed translucent solutions in water, and was precipitated on the addition of 2 volumes of ethanol. This precipitate was redissolved, insoluble protein and cystine removed by centrifugation, and the material made up to 1 per cent solution and the experiment repeated. It was attacked by the phosphorylase at the same rate as the original glycogen.

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[2] Cori, G. T., unpublished.

The results in Fig. 2 indicate that muscle phosphorylase can degrade glycogen and amylopectin beyond the branch points. As a further check the limit dextrin of glycogen was prepared by prolonged action of β-amylase. This material was incubated for 24 hours with a phosphate-phosphorylase-phosphoglucomutase system. (The latter enzyme was added in order to convert the glucose-1-phosphate formed by phosphorylase to glucose-6-phosphate and thereby to shift the equilibrium away from glycogen.) The barium salts of the phosphate esters were isolated in good yield and analyzed for total and easily hydrolyzable phosphate. In three such experiments the easily hydrolyzed phosphate averaged 5 per cent of the total esterified phosphate. This is the proportion found when phosphoglucomutase acts on a solution of pure glucose-1-phosphate. Since the limit dextrin was used, the formation of phosphate esters indicates that muscle phosphorylase can by-pass 1,6 linkages.

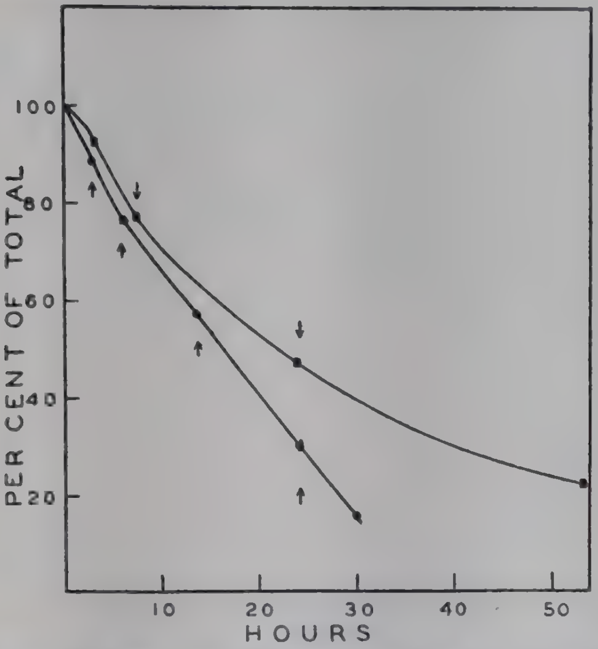

Fig. 2. Degradation of branched polysaccharides by crystalline muscle phosphorylase. Value plotted as a fraction of the amount of carbohydrate found in the control; ●, glycogen; ■, amylopectin; the arrows represent the addition of more enzyme.

The question whether phosphorylase can actually break 1,6 linkages has not been settled. When dextran was treated with muscle phosphorylase, no uptake of phosphate was found even on prolonged incubation with an amount of enzyme which completely degraded a similar amount of glycogen in 3 hours. However, as in the case of α-amylase, this negative result is open to the criticism that the structure of the dextran may be so different from that of glycogen as to be unavailable to attack. Interestingly enough, dextran is able to activate phosphorylase for polysaccharide synthesis (Paper III).

Potato phosphorylase is similar to β-amylase in being unable to pass the branch point. Meyer and Bernfeld (8) have shown that the limit dextrin from amylopectin is not attacked by potato phosphorylase.

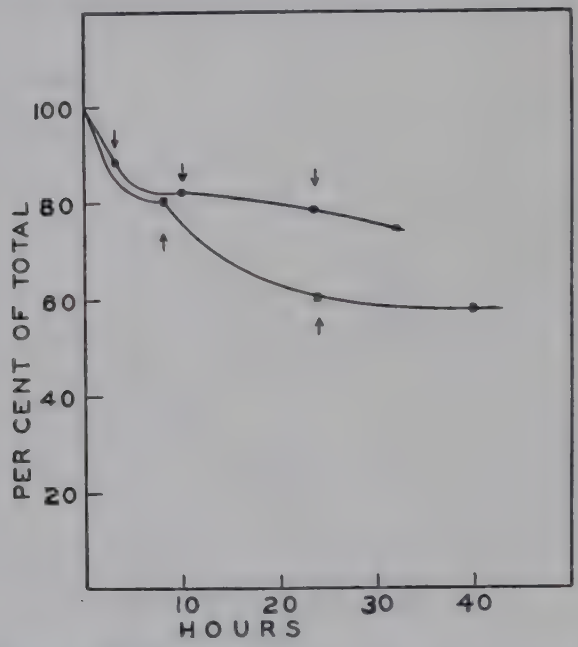

Fig. 3. Degradation of branched polysaccharides by potato phosphorylase. ●, glycogen; ■, amylopectin; the arrows represent the addition of more enzyme.

When amylopectin and the potato enzyme were dialyzed against a phosphate buffer, as described for the muscle enzyme, the degradation did not proceed beyond 40 per cent, as compared to a degradation of 60 per cent with β-amylase. The ability of the potato enzyme to degrade glycogen was even more limited (see Fig. 3).

The following experiment was designed to characterize the limit dextrin formed from amylopectin by potato phosphorylase. About 250 mg. of amylopectin and a concentrated solution of potato phosphorylase were dialyzed for 40 hours against several changes of phosphate buffer, and then the residual polysaccharides precipitated with methanol. A portion of the dried sample was redissolved and treated with phosphorylase for a further 40 hours. This second residue was also precipitated and dried. The two fractions were tested for their ability to activate potato phosphorylase in the synthesis of polysaccharide and for their susceptibility to attack by β-amylase. The results are shown in Table III. It is seen that even at the limit of degradation with potato phosphorylase some branches remain which can be removed by the β-amylase. These remaining branches, perhaps 5 to 6 units long if the branches were shortened uniformly, are not very effective in activating phosphorylase in the synthetic reaction. The limit dextrin formed from amylopectin by β-amylase does not activate potato phosphorylase.

TABLE III

Properties of Residue from Amylopectin Degraded with Potato Phosphorylase

Residue I, obtained after 40 hours of incubation with potato phosphorylase, was isolated and incubated with the enzyme for another 40 hours, giving Residue II. Both residues were tested for their ability to activate potato phosphorylase for polysaccharide synthesis with glucose-1-phosphate as the substrate. An amount of residue was used corresponding to an original amylopectin concentration of 35 mg. per cent and was compared with the activating effect of 500 mg. per cent of soluble starch. Both residues were also subjected to the action of β-amylase.

Sample	Time of degradation	Activating effect for phosphorylase	Degradation by β-amylase
	hrs.	*per cent*	*per cent*
Amylopectin	0	70	60
Residue I	40	62	30
" II	80	38	19

SUMMARY

The degradation of polysaccharides by four enzymes has been investigated. The action of salivary α-amylase on amylose, a linear polysaccharide, appears to be more random than its action on glycogen, a branched polysaccharide. During the action of β-amylase of wheat on amylose, intermediates of short chain length could not be detected. This observation, as well as others, suggests that this enzyme degrades one whole amylose molecule completely to maltose before attacking a new chain. Both α- and β-amylase hydrolyzed the short saccharide chains obtained by partial hydrolysis of the Schardinger dextrins.

Muscle phosphorylase can degrade glycogen and amylopectin beyond the branch points. Potato phosphorylase is similar to β-amylase in being unable to pass branch points, but differs from β-amylase in removing the terminal chains of amylopectin less completely. No evidence has been obtained that any of these enzymes can break the α-1,6 linkages at the branch point. They do not act on dextran, a polysaccharide consisting of α-1,6 linkages.

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STUDIES ON THE STRUCTURE OF POLYSACCHARIDES

I. RELATION OF STRUCTURE TO ACTIVATION OF PHOSPHORYLASES*

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Since it was discovered that the synthesis of polysaccharide by phoshorylase would not proceed in the absence of a small amount of preformed olysaccharide (1), there has been much interest in the nature and function f the so called activator or primer. The explanation best in accord with bserved facts is that the activator furnishes end-groups which act as uclei for the addition of new chain units (2). Muscle phosphorylase pparently requires a large molecule with many end-groups, qualities best upplied in glycogen. In contrast, potato phosphorylase is activated by nany starch-like materials (3–5), the structural requirements being much ess exacting than those of the muscle enzyme. The small amount of lextrin which usually contaminates maltose and enzymatically prepared glucose-1-phosphate causes activation of the potato enzyme, while recrystallized maltose and synthetically prepared glucose-1-phosphate have no activating effect. Recently, Proehl and Day (6) reported that bacterial lextran had slight activating ability.

In a preliminary report (7) it was shown that dextrins averaging 4 to 5 glucose units in length could activate potato phosphorylase. In the present paper this investigation will be reported in greater detail. The relation of activation to the size of the dextrins was studied by observing changes in the activating ability when polysaccharides were hydrolyzed by various agents. In this connection some observations have also been made on the structure of branched polysaccharides.

EXPERIMENTAL

The acid hydrolyses were carried out as described in Paper I of this series. For the enzymatic hydrolysis, 100 mg. per cent solutions of polysaccharide in 0.02 M citrate at pH 6.5 were treated with enough enzyme to

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effect about 50 per cent hydrolysis in 1 hour.[1] At suitable intervals samples were removed and heated at 100° for 10 minutes to stop enzyme action. Usually the determination of the activating and the reducing power was made on the same day as the hydrolysis.

For testing the activating ability a polysaccharide concentration of 30 to 35 mg. per cent was chosen, since it has been found that 12 to 15 mg. per cent of amylopectin gave half maximal activation of potato phosphorylase. At double this concentration the system was still sensitive to increases in end-groups, while the activity in the presence of less effective substances was not too small to be measured. The material to be tested was substituted for the starch or glycogen in the standard test system, which consisted of equal parts of polysaccharide solution, 2.25 per cent glucose-1-phosphate (as dipotassium salt), and enzyme. The phosphorylase of potato was diluted with 0.1 M citrate buffer at pH 6.5, and that of muscle with 0.03 M cysteine-1 per cent sodium glycerophosphate at pH 6.8.[2] After 5 minutes incubation at 30°, the reaction was stopped by addition of part of the sulfuric acid needed for the phosphate determination by the method of Fiske and Subbarow (9). Each series contained a standard tube containing 500 mg. per cent of starch or glycogen, and all activations were calculated as a percentage of this maximal value. Because of the great sensitivity of the muscle enzyme to salts, the chloride concentration of the standard glycogen solution was adjusted to that of the test solutions. Synthetic glucose-1-phosphate was used throughout, since the enzymatically prepared ester always gives a substantial activity with the potato enzyme, a factor which makes the quantitative relationships more difficult to evaluate.

The reducing power was measured with Shaffer and Somogyi's Reagent 60 (10), with a boiling time of 30 minutes. For acid hydrolysis it was expressed as per cent conversion to glucose and for the hydrolysis with amylase as per cent conversion to maltose, although maltose is not the sole reducing substance present.

Correlation of Activating Ability with End-Groups and Chain Lengths—The curves obtained by plotting the per cent activation against the per cent hydrolysis for acid-treated amylose and Schardinger dextrins are shown in Fig. 1. Each curve is the composite of several series of determinations. The characteristic rise and fall of the activating ability is a function of the number and size of the fragments formed during the hydrolysis. Two opposing effects are in operation; as hydrolysis progresses, more and more end-groups become available for the activation, but at the same time some chains become too short to be activating. The curve for the α-dextrin is lower and flatter than that of the β-dextrin because the slower rate of ring opening (Paper I) leads to a smaller but more prolonged supply of open chains.

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[1] β-Amylase was prepared by the method of Ballou and Luck (8) from wheat. Diluted saliva was the source of α-amylase. Extra NaCl, 400 mg. per cent, was added to the substrate of α-amylase.

[2] The crystalline muscle phosphorylase was prepared by Dr. G. T. Cori from rabbit muscle. The potato phosphorylase was prepared by Dr. A. A. Green. It had an activity of 10 to 15 units per mg. of protein, when 1 unit is the amount of enzyme liberating 0.1 mg. of phosphorus in 5 minutes at 30° and pH 6.5 in the presence of 500 mg. per cent of starch and 750 mg. per cent of glucose-1-phosphate. It was free of phosphatase and amylase activity.

The lengths of the chains available for activation were determined after glucose and maltose were removed by fermentation with yeast. Then the reducing power was determined before and after complete acid hydrolysis, and the data used for the calculation of the average chain length. In the case of the Schardinger dextrins the unopened rings had also to be determined (Paper I). With both amylose and the β-Schardinger dextrin the peak of activation occurred when the average length of the chains was 4 to 5 glucose units, and this was also the average maintained over the flat part of the curve for the α-dextrin. The fermented hydrolysate from amylose at the peak of the curve is a relatively homogeneous mixture, containing no fragments over 7 (since it is achromic to iodine) nor under 3 units long. It should prove a good material from which to isolate fractions to study the minimal chain length required for activation.

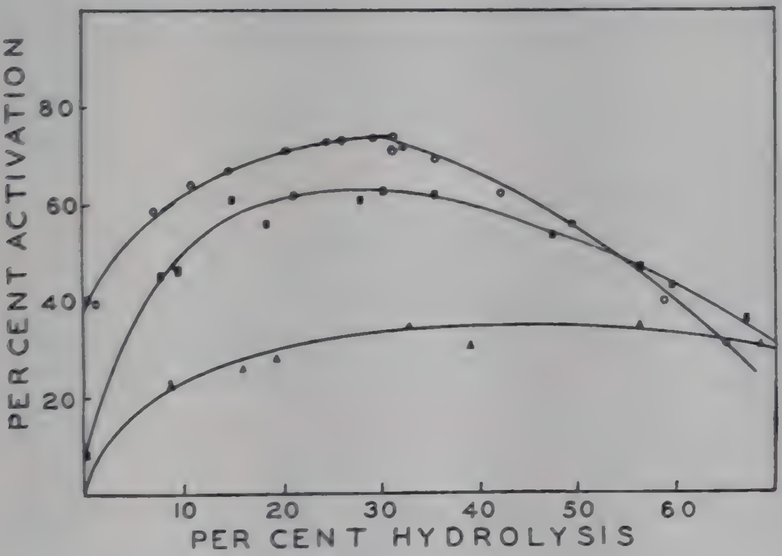

Fig. 1. Activation of potato phosphorylase by acid-hydrolyzed unbranched polysaccharides. The average chain length at the peak of activation was between 4 and 5 units. See the text. ○, amylose, ■, β-Schardinger dextrins, ▲, α-Schardinger dextrin.

Weibull and Tiselius (11) separated dextrins of various chain lengths with the aid of their adsorption analysis technique. They found pure maltose to be inactive, while tri- to hexasaccharides had an activating effect for potato phosphorylase. There is thus fair agreement as to the requirements of potato phosphorylase for activation. This enzyme is useful as an indicator for the presence of dextrins too short to give an iodine color.

Of some importance is the question of the affinity of these short chains for phosphorylase as compared to starch or amylopectin. It should be emphasized again that all comparisons of activating ability in the experiments in Figs. 1 to 7 were made with equal concentrations of polysaccharides expressed as glucose. For an equal concentration, Weibull and Tiselius found a hexasaccharide to be more activating than starch. According to the present theory, a comparison of activating ability should be based on the concentration of activating end-groups. According to our measurements, starch and amylopectin with about 4.5 per cent end-groups give half maximal activation of potato phosphorylase at a concentration of end-groups of about 3×10^{-5} M per liter. A similar value is found for corn amylose, its average chain length being assumed to be about 200. For dextrins of an average chain length of 5 units (derived from hydrolysis of either amylose or β-Schardinger dextrin) the corresponding value was 1.4×10^{-4} M per liter. This indicates that per mole of end-group the short chains are less effective as activators than the native polysaccharides. A recalculation of the data of Weibull and Tiselius gives the same result.

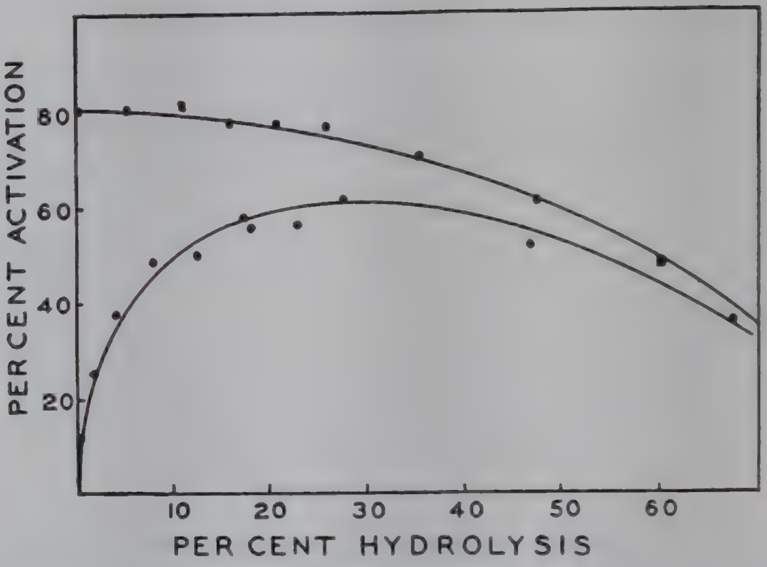

FIG. 2. Activation of potato phosphorylase by acid-hydrolyzed branched polysaccharides. ■, amylopectin, ●, amylopectin limit dextrin.

The curves obtained with several polysaccharides hydrolyzed with different agents are presented in Figs. 2 to 6. Acid hydrolysis produces a rise in activation in every case except that of amylopectin. Hydrolysis by α-amylase causes a small increase, even with amylopectin. The β-amylase always decreases the activation. In all cases the curves were reproducible and quite characteristic, so that this method can be used as a means of identifying an unknown polysaccharide of the group tested. This is shown in a previous paper in which the activating effects of a number of additional polysaccharides have been tabulated (7).

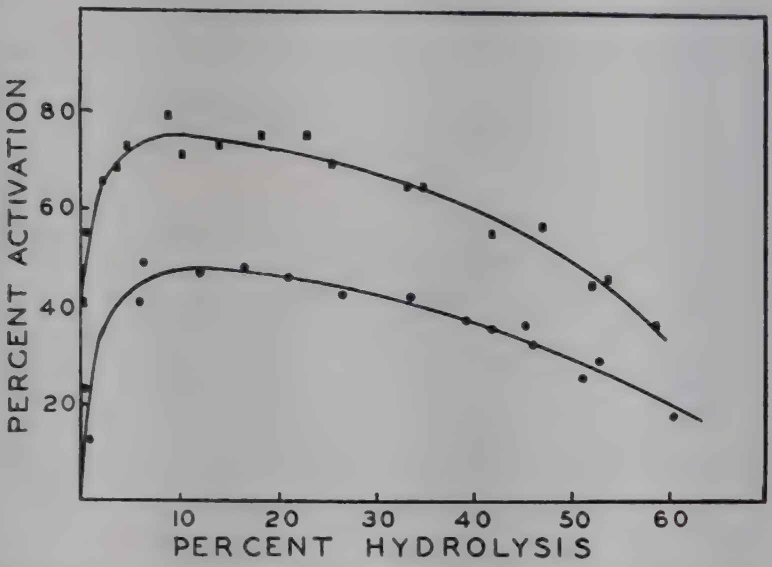

FIG. 3. Activation of potato phosphorylase by acid-hydrolyzed branched polysaccharides. ■, glycogen, ●, glycogen limit dextrin.

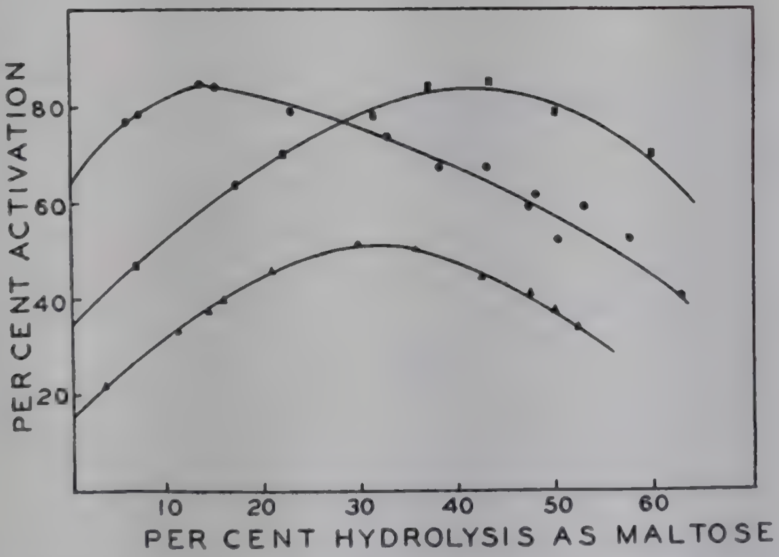

FIG. 4. Activation of potato phosphorylase by polysaccharides hydrolyzed by α-amylase. ●, amylopectin, ■, amylose, ▲, glycogen.

In general, the increase or decrease in activating ability observed in these experiments is in harmony with the concept that activation depends on the number of end-groups available, provided the chains to which the end-groups are attached are above a certain minimal size. Amylopectin seems to be an exception, since it is difficult to explain why acid hydrolysis does not result in an increase in available end-groups. The limit dextrin of amylopectin shows a large increase in activating ability on acid hydrolysis (Fig. 2). Since this is due to the breaking up of the inner structure, it

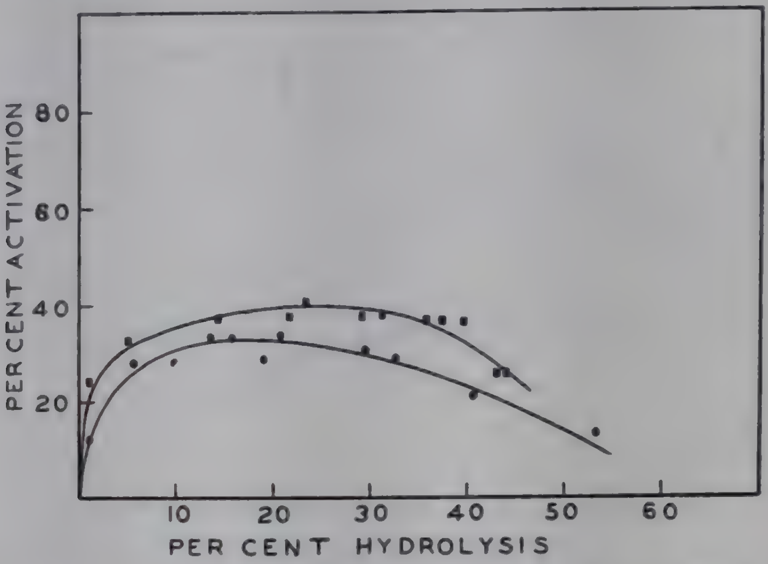

FIG. 5. Activation of potato phosphorylase by limit dextrins hydrolyzed by α-amylase. ●, amylopectin limit dextrin, ■, glycogen limit dextrin.

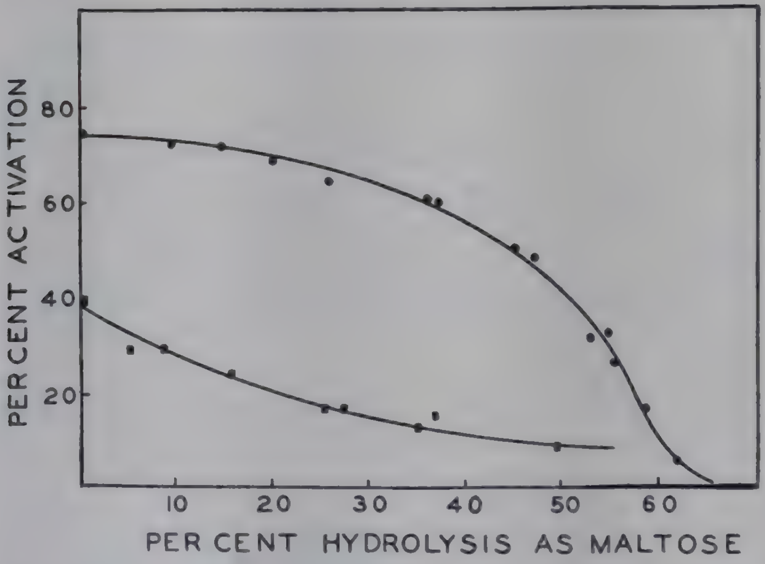

FIG. 6. Activation of potato phosphorylase by polysaccharides hydrolyzed by β-amylase. ●, amylopectin, ■, amylose.

would appear that acid causes a decrease in the activating ability of the outer structure faster than the inner segments are made available.

The very low activating ability of the limit dextrins of amylopectin and glycogen (Figs. 2, 3, and 5) shows that an end-group must be more than 1 or 2 units from a branch point in order to be available to potato phosphorylase. Actually, if we judge from the data of Table III, Paper II, a somewhat longer chain may be required. The fact that the effective chain length of the outer structure is limited by the branch point makes it possible to explore the inner structure of branched polysaccharides by means of potato phosphorylase.

The hydrolysis activation curves of the limit dextrins in Figs. 2, 3, and 5 show that both acid and α-amylase can split the inner structure in such a manner that activating chains are formed. If a chain of 3 to 4 units from a branch point is required and acid hydrolysis is random, then at least some of the inner segments must be as long as 6 to 8 units to be split to activating chains. According to an estimate of Meyer (12) the inner segments of amylopectin are on an average 8 to 9 units long. The evidence from phosphorylase activation is in agreement with this estimate. In the case of glycogen the inner segments were estimated to be on an average only 3 units long (12), and this is not compatible with the evidence from phosphorylase activation, unless one makes the assumption that the inner structure of glycogen is very irregular, consisting at least of some long inner segments.

That the 1,6 linkage itself does not interfere with activation is shown by the fact that bacterial dextran activates potato phosphorylase. Proehl and Day (6) found that 570 mg. per cent of dextran dissolved in water had about 40 per cent of the activating effect of 150 mg. per cent of amylopectin. Similar observations have been made in this laboratory with two different samples of dextran. When high concentrations of the dextrans were dispersed in water or in 7.7 N HCl (followed by neutralization), the activation was about 30 per cent of maximum. Dextran dissolved in high concentration in 3 N KOH, followed by neutralization, was as effective an activator as the same amount of starch. At lower concentrations the dextran was less effective than starch. When more dilute alkali was used, the activating effect was less than that of starch even in high concentrations. Apparently the effect is one of degree of dispersion, the higher alkalinity bringing the dextran into more perfect solution. An effect of alkali on the solubility of dextran has been described by Daker and Stacey (13) and may be connected with the elimination of nitrogenous impurities. That the degree of dispersion plays a rôle has also been shown in the case of amylose, which is more effective as an activator when first dissolved either in acid or alkali than when dispersed in water.

Although each glucose unit in the dextran has a free hydroxyl on carbon 4, only a few of these seem to act as primers of phosphorylase. Correlation of the iodine color of the newly formed polysaccharide with the amount of glucose-1-phosphate that has reacted should give an estimate of the number of activating groups used in the dextran. In one sample studied spectrophotometrically, the spectrum of the iodine complex of the newly formed polysaccharide closely resembled that of amylose. From data to be presented in Paper IV one could estimate that the newly formed chains were at least 50 units long. Since 2.36×10^{-3} mM of glucose-phosphate had reacted, the concentration of activating groups should have been one-fiftieth of that value or 4.7×10^{-5} mM, as compared to a glucose concentration in the dextran of 1.4×10^{-2} mM. This would indicate that only 0.34 per cent of the hydroxyls on carbon 4 was involved in activation, a value which is even smaller than the number of end-groups found for this sample of dextran.[3]

Treatment of amylose or of amylopectin with β-amylase results in a decrease in activating ability (Fig. 6). If all chains were shortened uniformly, the moles of end-groups would remain the same and the decrease in activating ability would result from decreased affinity. Another possibility is that suggested in Paper II, namely that a whole chain is completely degraded before a new one is attacked. In this case the decrease in activating ability would result from a loss of end-groups. Calculations based on the second assumption show fair agreement in the case of amylopectin. In the case of amylose the loss of activating ability is greater than can be explained by the loss of end-groups, perhaps because β-amylase attacks preferentially the short amylose chains.

The requirements of muscle phosphorylase are narrower than those of the potato enzyme. Glycogen is the best activator, and its effectiveness increases slightly after very short hydrolysis in 6 N HCl at 30° (7), followed by a sharp decrease. In 7.7 N HCl (Fig. 7) the activating ability of glycogen decreases rapidly from the start, in contrast to its greatly increased effectiveness for potato phosphorylase (Fig. 3). Hydrolysis of glycogen by α-amylase (Fig. 7) results in a slight initial increase in activating ability (see also (15)); with more prolonged action of α-amylase the activating ability is lost rapidly. Glycogen limit dextrin prepared by β-amylase has some activating effect, while that of amylopectin is only slightly active. Hydrolysis of the former material only decreases the activation (Fig. 7). These observations and those previously recorded (7) all agree with the concept that muscle phosphorylase requires a large molecule for activation.

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[3] The dextran used was that analyzed by Hassid and Barker (14) and found to yield about 2.9 per cent tetramethyl glucose.

Dextran dissolved in high concentrations in alkali and then neutralized had nearly the same activating ability for muscle phosphorylase as glycogen, but on dilution activation decreased rapidly. When dissolved in acid, dextran hardly activated even in high concentrations, and activation disappeared on hydrolysis. Since dextran cannot be split by phosphorylase in the presence of phosphate (Paper II), this represents an example of a molecule which can activate both potato and muscle phosphorylase but cannot be attacked by them.

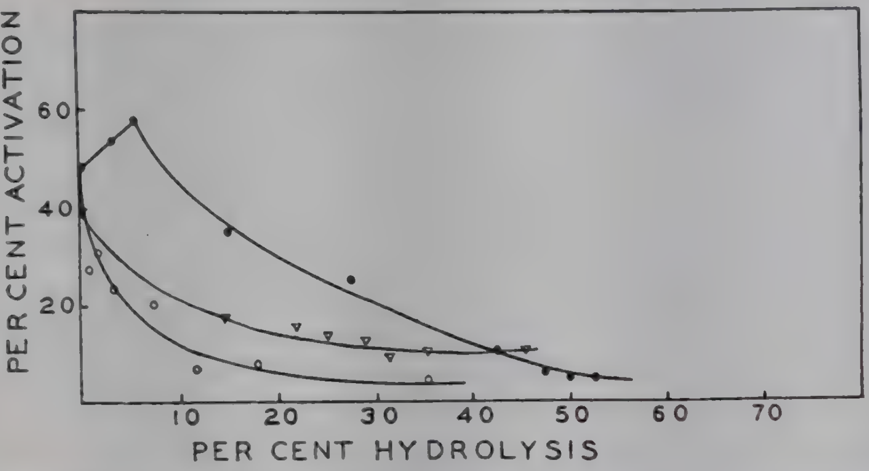

FIG. 7. Activation of muscle phosphorylase by hydrolyzed polysaccharides. ●, glycogen and α-amylase, △, glycogen limit dextrin and α-amylase, ○, glycogen in 7.7 N HCl.

SUMMARY

1. The hydrolysis products of various polysaccharides were tested for their activating ability for potato phosphorylase. When the per cent hydrolysis was plotted against per cent activation, curves were obtained which were characteristic for each polysaccharide and for the hydrolytic agent used (acid or α- or β-amylase). The increase or decrease in activation was shown to depend on the number of end-groups formed during hydrolysis as well as on the size of the fragments. Chains only 4 to 5 glucose units long, derived from hydrolyzed amylose or Schardinger dextrins, had activating power for potato phosphorylase.

2. A study of the activating effect of branched polysaccharides during hydrolysis by β-amylase shows that the branch point limits the length of the chain and that potato phosphorylase cannot be activated by an end-group less than 3 or 4 units removed from a branch point.

3. The limit dextrins of amylopectin and glycogen can be hydrolyzed by α-amylase or acid to yield activating fragments. This shows that some of the inner segments of these polysaccharides are at least 6 to 8 glucose units long.

4. Muscle phosphorylase is activated only by large soluble molecules with many end-groups, such as glycogen and amylopectin. Enzymatic or acid hydrolysis causes a rapid loss of activating power.

5. Bacterial dextran which contains primarily α-1,6 linkages, when dissolved in alkali in high concentration and then neutralized, is able to activate both potato and muscle phosphorylase.

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STUDIES ON THE STRUCTURE OF POLYSACCHARIDES

IV. RELATION OF THE IODINE COLOR TO THE STRUCTURE*

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There has been a large amount of work done on the blue colored complexes which starch forms with iodine (*cf.* the review by Barger (1)). When a sharp fractionation of starch was achieved, it was found that amylose contributed most of the blue color, that of amylopectin being a comparatively pale lavender. Since amylopectin is much larger and more colloidal in nature, it seems that the color formed with iodine is related to the structure of the molecule rather than to its colloidal properties. Evidence has been presented by Rundle and French (2) that amylose in solution has a helical structure and that the iodine molecules are held lengthwise within the helix.

There have been comparatively few quantitative studies of the spectrum of the complex. In 1938 Hanes and Cattle (3) studied the relation of the color changes to the development of reducing power during the hydrolysis of starch by different amylases. They discussed at length the possible interpretations for the changes observed, and the relation of iodine color to structure as it was then regarded. Simerl and Browning (4) and to a greater extent Kerr and Trubell (5) have studied the spectra of starch fractions.

In the present investigation the changes in the polysaccharide-iodine spectrum were studied during hydrolysis of amylose by various agents and during synthesis of polysaccharide by phosphorylase. It was found that the hue of the complex is dependent only on the length of the unbranched chains of the polysaccharide. Mixtures of different polysaccharides show that each component has an additive effect on the production of the final color. Changes in the spectrum during enzymatic hydrolysis give important information on the mode of action of the enzymes. Consideration of the results of Hanes and Cattle in the light of the newer knowledge of the

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properties of the starch fractions shows that our results are in complete agreement with theirs.

EXPERIMENTAL

Since salts have been observed qualitatively to affect the color, the concentration of salts was kept as low as possible (about 0.001 M) and the ratio of polysaccharide to salt approximately constant. The acid and enzymatic hydrolyses were carried out as described in Papers I and II. For synthesis by means of phosphorylase a solution of an achroodextrin of known average length was used as the activator. At intervals, a drop was removed and treated with iodine. When a color change was detectable, a measured sample was removed and the reaction was stopped with dilute sulfuric acid. Aliquots of this solution were used for both phosphate determinations and spectrophotometric studies. The final sulfuric acid concentration in all solutions used for study of the spectra was 0.01 N. The observations were made in a Beckman spectrophotometer.

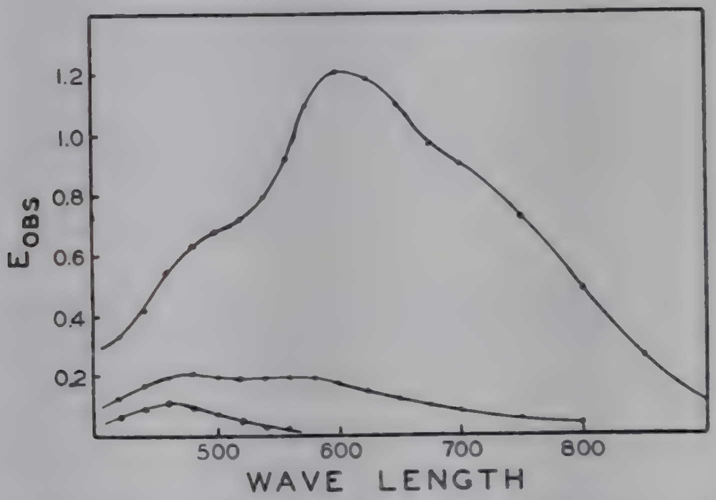

Fig. 1. The absorption spectra of amylose and amylopectin in the presence o iodine with no added iodide. Upper curve, amylose; middle curve, amylopectin lowest curve, iodine alone. The polysaccharide is in excess.

Effect of Iodine on Spectrum—Hanes and Cattle found that the color of the starch-iodine complex was added to that of the iodine alone. We found this to be true even when the polysaccharide was in excess. In Fig. 1 are shown the spectra of 10 mg. per cent solutions of amylose and amylopectin to which a saturated solution of iodine in water has been added. The lowest curve is that of the iodine alone. The excess of polysaccharide was so great that the intensity of the color was not at all changed by doubling the concentration of the polysaccharide, but nevertheless the

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small peak given by iodine at 450 mμ was plainly visible in the curves for the two polysaccharides.

When iodide ion is added, this peak shifts toward the ultraviolet and only a small portion of it is visible at wave-lengths longer than 400 mμ. In Fig. 2 are the spectra of 5 mg. per cent amylose and amylopectin in the presence of 10 mg. per cent iodine and 20 mg. per cent potassium iodide. The lowest curve is that of the iodine-iodide alone and is essentially the same as that found by Hanes and Cattle.

In Figs. 3 and 4 are the spectra of various concentrations of amylose and amylopectin when the absorption due to the iodine has been deducted. Here the density is proportional to the concentration of the polysaccharide.

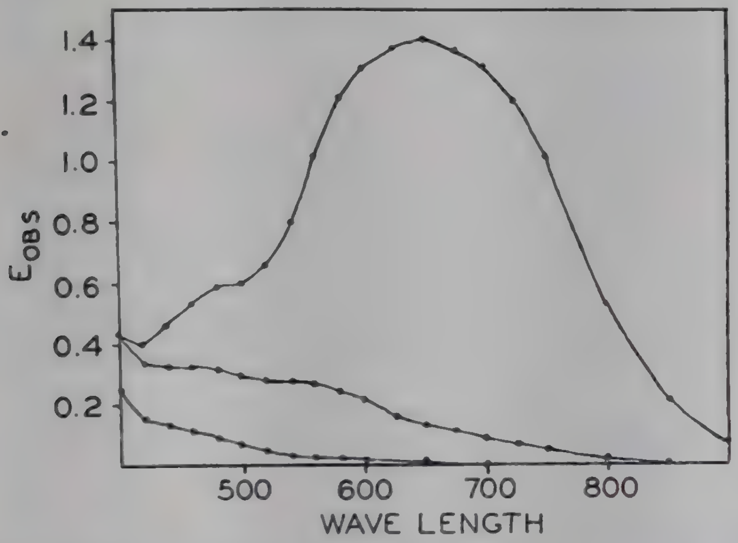

FIG. 2. The absorption spectra of amylose and amylopectin in the presence of iodine-iodide. Upper curve, 5 mg. per cent amylose; middle curve, 5 mg. per cent amylopectin; lowest curve, 10 mg. per cent iodine, 20 mg. per cent potassium iodide. The iodine is in excess.

Unless otherwise specified, all observations reported were made in the presence of excess iodine-iodide and the absorption due to the iodine blank was deducted from that observed for the polysaccharide-iodine mixtures to obtain that due to the complex alone.

Spectra of Starch Fractions—The amylose-iodine spectrum was found to have a broad, symmetrical peak around a maximum at 650 mμ (Fig. 3). Amylopectin formed a complex having a much feebler absorption with a peak at 540 mμ (Fig. 4). The previously reported maxima for amylose were at 600 to 620 mμ. This probably shows that the amylose fraction was not entirely freed of amylopectin, since the addition of amylopectin to amylose shifts the peak toward the shorter wave-lengths. This is shown in

Fig. 5 where the curves obtained for soluble starch and a 1:4 amylose-amylopectin mixture are compared. The middle curve is that obtained b adding independent curves for the two fractions. These curves are al similar in general shape and location of the peak, and show that the effec

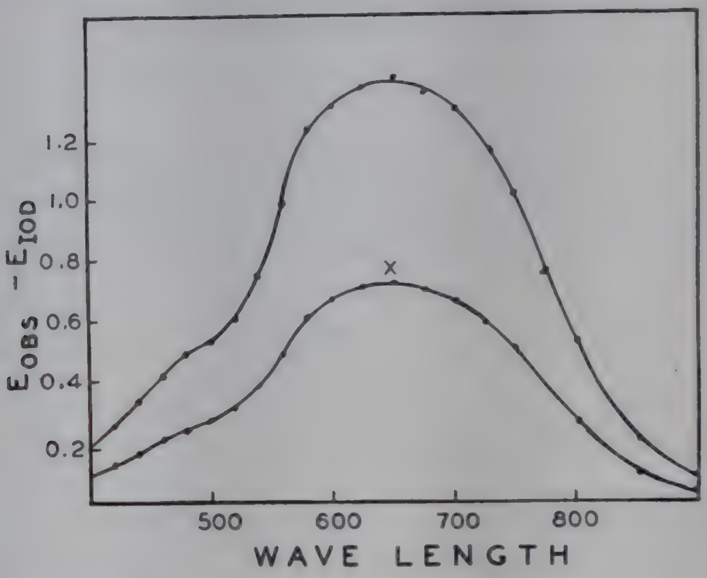

FIG. 3. The absorption spectra of the amylose-iodine complex, observed density minus density of the iodine blank. Concentration of amylose 5 and 2.5 mg. per cent.

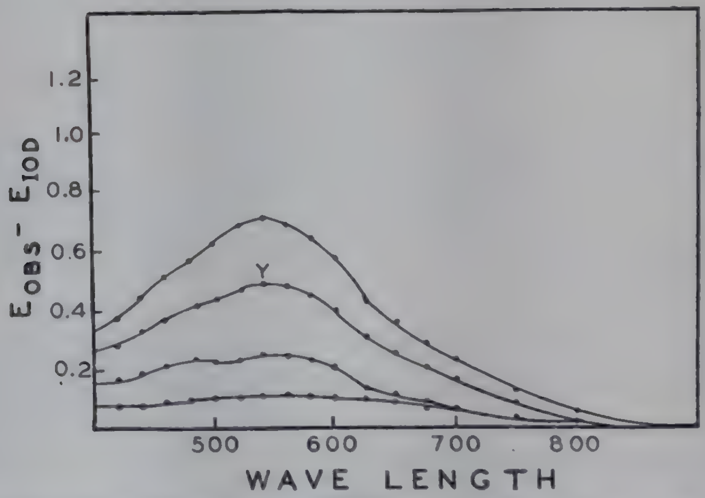

FIG. 4. The absorption spectra of the amylopectin-iodine complex, observed density minus density of the blank. From the top down the concentrations of amylopectin are 15, 10, 5, and 2.5 mg. per cent.

of two differently staining polysaccharides is additive. The shape of the starch curve beyond 650 mμ is similar enough to that of amylose to justify using the density at 660 mμ as an approximate measure of the amount of amylose in starch.

Effects of Hydrolysis—Random hydrolysis by acid shifts the peak of bsorption toward the short end of the spectrum mainly by decreasing bsorption in the longer wave-lengths (Fig. 6). The topmost curve in ig. 6 shows that, even though measurable reducing power had not appeared in the solution (8 minutes in 7.7 N HCl at 30°), the absorption spectrum had already been changed. When α-amylase was the hydrolytic agent, the color shift was similar to that in acid (Fig. 7). The main difference lies in the greater shift toward the red in proportion to the decrease in

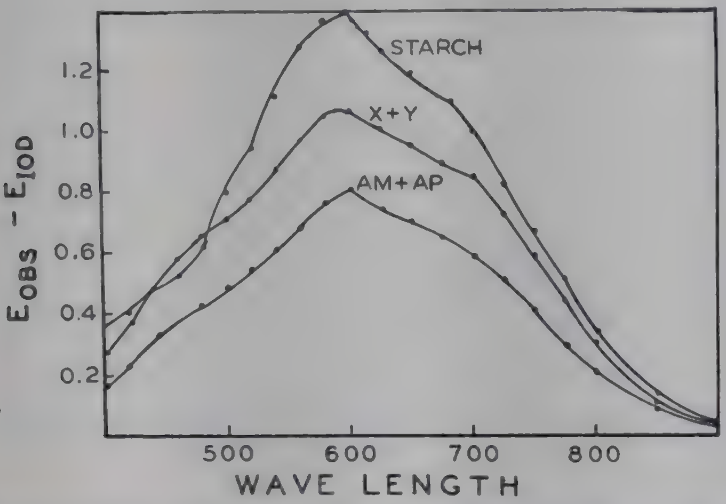

FIG. 5. Absorption spectra of mixtures of starch fractions. Upper curve, soluble starch; middle curve, a composite of independent curves for amylose and amylopectin, marked *X* and *Y* in Figs. 3 and 4 respectively; lowest curve, a mixture of amylose and amylopectin 1:4.

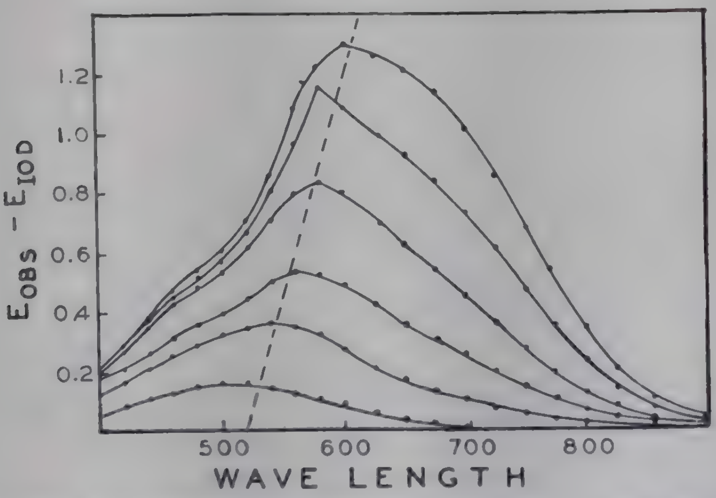

FIG. 6. Changes in the absorption spectra of amylose during hydrolysis in 7.7 N HCl at 30°. Progression from the top downward, corresponding to 8, 18, 30, 60, 90, and 150 minutes of hydrolysis.

the amount of color. This probably indicates that enzyme hydrolysis is not completely random, but that fragments of certain sizes are preferentially removed (*cf.* Paper II).

An entirely different picture is obtained with β-amylase (Fig. 8). Hanes also found that under the influence of this enzyme the form of the curve remained essentially that of the original starch. This same effect was reported by Bourne, Macey, and Peat (6) in a slightly different fashion. They found that the relation of the "blue value," or absorption at 660 mμ,

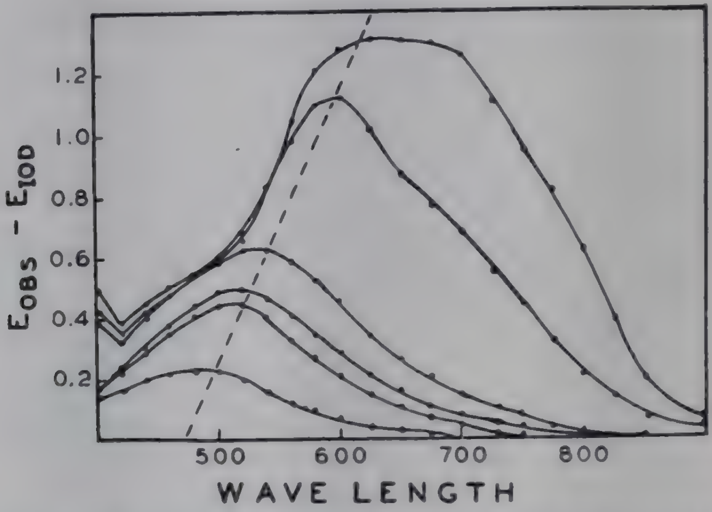

FIG. 7. Changes in absorption spectra of amylose during hydrolysis by α-amylase. Progression from the top downward, corresponding to 30 per cent hydrolysis for the last curve.

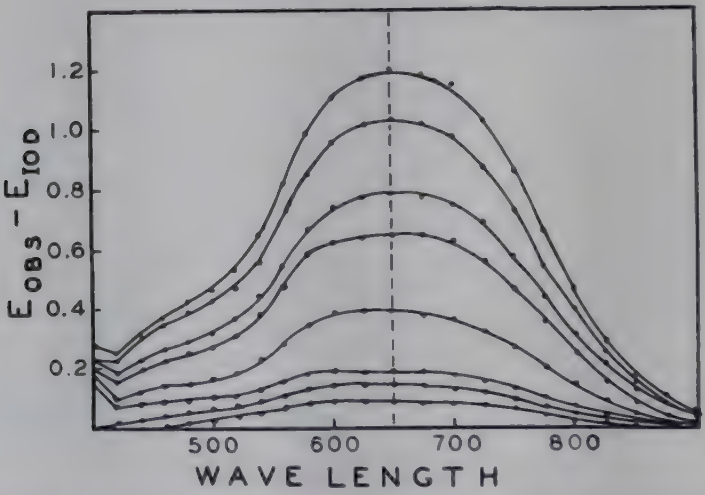

FIG. 8. Changes in absorption spectra of amylose during hydrolysis by β-amylase. Progression from the top downward, corresponding to 91 per cent hydrolysis for the last curve.

to reducing power was a straight line when amylose was the substrate. The decline in the intensity of absorption without any shift in the peak or change in the shape of the curve shows that during action of β-amylase the amount of amylose decreases without the formation of any iodine-staining

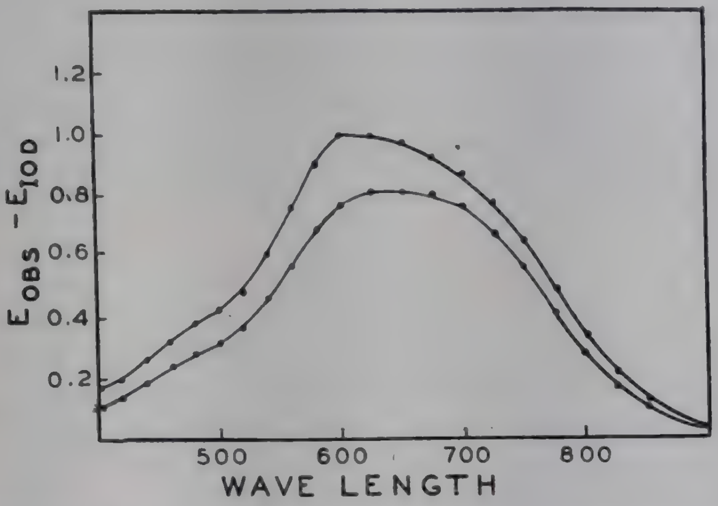

Fig. 9. Absorption spectra of amylose, and amylose-erythrodextrin mixture. Upper curve, 2.5 mg. per cent amylose and 2.5 mg. per cent dextrin; lower curve, 2.5 mg. per cent amylose.

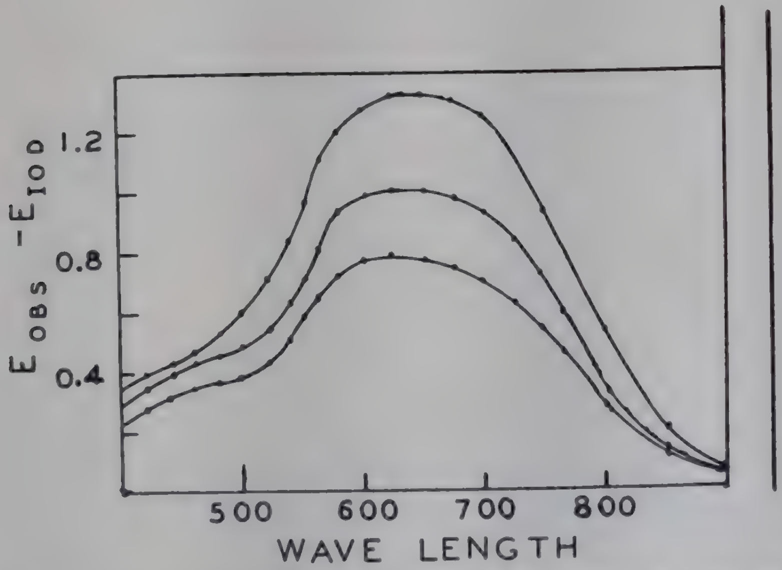

Fig. 10. Changes in absorption spectra during degradation of amylose by potato phosphorylase.

intermediates. The addition of a red-purple staining dextrin to amylose actually shifted the peak (Fig. 9). This shows that the intensity of color due to the amylose is not so great as to obscure completely the effect of the less intensely staining material. Potato phosphorylase also degrades amylose with no change in the spectrum (Fig. 10).

Relation of Iodine Color to Chain Length—From the results obtained with hydrolyzed amylose it appears that the color is related to the length of the chains. In order to test this, a series of molecules of known size is necessary. One way of obtaining these is through synthesis by means of phosphorylase. If an achroodextrin solution with a known number of end-groups is used as activator, the size of the synthetic polysaccharide can be calculated from the inorganic phosphate liberated, as shown in Tables I and II. This gives a minimum value since it involves the assumption that the added units are distributed uniformly among the dextrin end-groups, which may not be strictly correct because some of the dextrins may be too small to activate.

TABLE I

Chain Lengths of Synthetic Polysaccharides

Activating dextrin prepared by acid hydrolysis of β-Schardinger dextrin. Concentration of end-groups in activating dextrin = 1.1×10^{-4} M; glucose-1-phosphate (initial concentration) = 1.9×10^{-2} M. The curves for the absorption spectra corresponding to each chain length are shown in Fig. 11.

Time	Phosphate liberated		Total chain lengths	Visual color with iodine	Absorption peak
min.	*per cent total*	*M × 10^3*			*mμ*
1	0	0	3.8	None	
5	2.1	0.4	7.4	Faint red	500
9	5.3	1.0	12.9	Red	520
13.5	8.4	1.6	18.3	Lavender	540
18	9.5	1.8	20.2	Purple	560
25.5	14.7	2.8	29.3	Blue-purple	580
32	17.9	3.4	34.7	Blue	580
60	30.0	5.7	55.6	"	600
135	54.2	10.3	97.4	"	600
360	82.6	15.7	146.5	"	600

The similarity between the curves found during synthesis (Fig. 11) and in reverse order during acid hydrolysis (Fig. 6) is striking. In both cases the absorption peaks show a shift from 520 to 600 mμ, and hence the same intermediate colors are traversed as the reaction progresses, from red to blue as the chains are lengthened by synthesis and from blue to red as they are shortened by hydrolysis. In Fig. 12 the polysaccharide is in excess relative to the iodine and there was no added iodide, so that these curves are comparable to those in Fig. 1. Again there was a shift in the colors from red to blue, and the absorption curves resembled more and more that of amylose as the chains were lengthened by synthesis.

In the above experiments with linear polymers the chain length appears to be the main factor influencing the hue of the iodine complex. The data of Tables I and II show that no visible colors are formed by chains up to 7 units long, red color by chains of 8 to 13 units, and blue color by chains

TABLE II

Chain Lengths of Synthetic Polysaccharide

Concentration of end-groups in activating dextrin = 4.2×10^{-4} M; glucose-1-phosphate (initial concentration) = 2.66×10^{-2} M. The curves for the absorption spectra corresponding to each chain length are shown in Fig. 12. In contrast to the experiments in Fig. 11, no iodide was added and the polysaccharide was in excess relative to iodine.

Time	Phosphate liberated		Total chain length	Visual color with iodine	Absorption peak
min.	*per cent total*	*M × 10³*			*mμ*
1	3.0	0.8	5.7	None	
10	10.2	2.7	10.2	Red	520
15	15.0	4.0	13.3	Lavender	540
25	22.6	6.0	18.1	"	560
35	27.4	7.3	21.2	Purple	580
45	34.2	9.1	25.5	Blue-purple	580
95	46.2	12.3	33.1	Blue	600
135	53.0	14.1	37.4	"	600
240	54.5	14.5	38.3	"	600
480	75.9	20.2	51.9	"	600

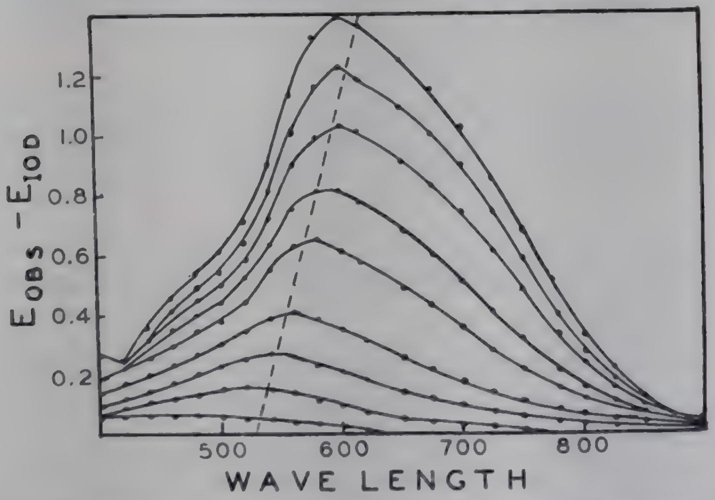

FIG. 11. Changes in absorption spectra during synthesis of the polysaccharide by potato phosphorylase. Progression from the bottom upward. For experimental details see Table I.

over 30 units. The relationship was checked by using solutions of amylose which had been partially hydrolyzed by α-amylase. The samples were dialyzed against large volumes of water to remove the very short chains;

then the chain lengths were determined from the reducing power as before. Three dialyzed solutions which stained red averaged 11 ± 0.5 units long, three that stained lavender were 21 ± 0.1 units long. End-group analysis of several dextrins has also shown that red iodine colors are formed by dextrins 8 to 12 units long (7).

The iodine colors can also be used to indicate the dialyzability of the dextrins. Some red staining chains formed by the hydrolysis of amylose can be dialyzed away in 20 hours (see Paper II, Table II); these had an average chain length of 8 units. Since red staining chains averaging 11 units were retained, the limit of dialyzability with the cellophane membranes used (Visking Corporation, NoJax) appears to be between 8 and 11 units.

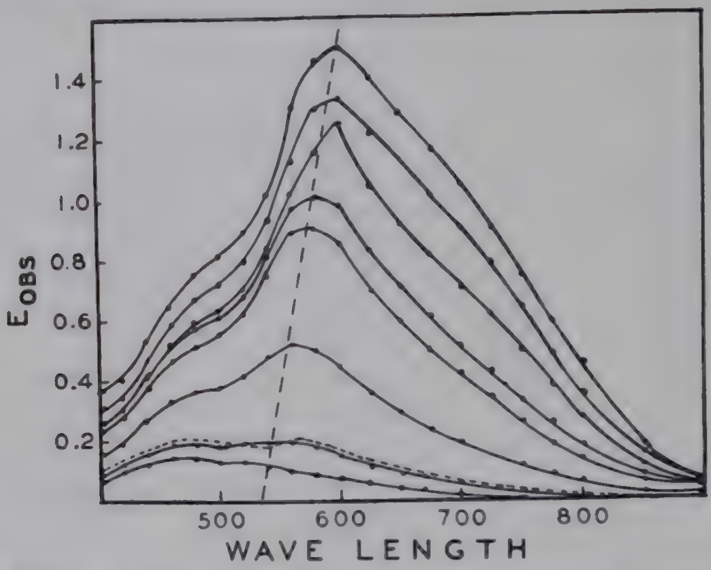

FIG. 12. Changes in absorption spectra during synthesis of polysaccharide by potato phosphorylase. No iodide is present, and the polysaccharide is in excess. The dotted line is that of amylopectin represented in Fig. 1. For experimental details see Table II.

The question arises whether the iodine color can be used as an index of the length of the segments in branched polysaccharides such as amylopectin. In the past, dependence of the color solely on the chain length has been doubted because materials were found having the same end-group assay, yet showing widely different colors. Two such cases are cited by Hanes and Cattle (3). A red staining non-retrograding dextrin was prepared by Haworth, Hirst, and Plant (7) by degrading starch in hot glycerol. After careful fractionation three methods of analysis showed it to be relatively homogeneous, and to average 11 to 12 units long. On the other hand, α-amylodextrin, which stains purple and retrogrades from solution, was also found by Haworth, Hirst, Kitchen, and Peat (8) to average 11 to 12 units. The amylodextrin was prepared by the action of β-amylase on unfractionated starch, and our present knowledge of the properties of the starch fractions and the action of β-amylase suggests that some undegraded amylose contaminated the limit dextrin and caused the blueness of the color as well as the tendency to retrograde. This can be demonstrated with purified fractions. In Fig. 13 are shown the spectra of amylopectin and amylose-amylopectin mixtures which had been treated with β-amylase until the reducing power showed no increase for 2 hours. The pure amylopectin limit dextrin had a red iodine color corresponding to a chain length of 10 units. The mixture containing amylose was distinctly lavender, and the curves show this to be due to the increase in absorption in the longer wave-lengths, which is as much as that expected from the curve in Fig. 8 corresponding to the same time of digestion (the third curve from the bottom).

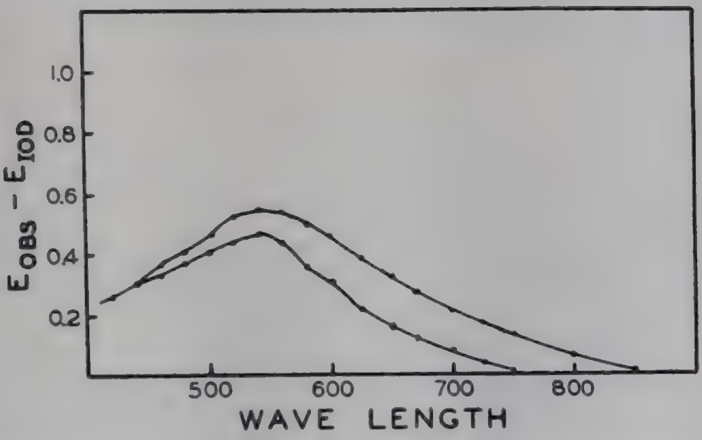

FIG. 13. Absorption spectra of amylopectin (lower curve) and amylose-amylopectin mixture 1:4 (upper curve), both digested with β-amylase.

Relation of Iodine Color to Structure of Branched Polysaccharides—The curve for amylopectin in Fig. 1 almost exactly corresponds to that of the 14 unit polysaccharide in Fig. 12; in order to show this, the curve for amylopectin has been superimposed and represented by a dotted line. This, however, does not mean that 14 is the longest chain which occurs in amylopectin, since shorter chains would redden the color. The limit dextrin of amylopectin has a color corresponding to a 10 unit chain. This agrees well with data obtained from activation experiments (Paper III) showing that some of the inner segments are that long. It was hoped that mixtures of limit dextrin with synthetic polysaccharides of different chain lengths would give some information on the lengths of the outer branches of amylopectin. The curves are shown in Fig. 14. The shape of the curve for amylopectin differs from that of the mixtures at the shorter wave-length, but from the location of the peaks and the absorption at longer wave-lengths it may be estimated that the outer branches of amylopectin are close to 18 units. Meyer (9) has estimated them to be 15 to 18 units on the basis of experiments with degradation by β-amylase.

It seems probable that the branch point effectively limits the length of the chain in forming the iodine complex. It is unknown whether branching has any other influence on the color formed. However, the results with amylopectin encourage the application of the same method to glycogen. The absorption curve of the glycogen-iodine complex resembles that of a dextrin about 8 to 9 units long. The limit dextrin has a very faint red coloration, suggesting that there are a few inner segments at least 8 units long. Again, this is in agreement with the data from the activation experiments.

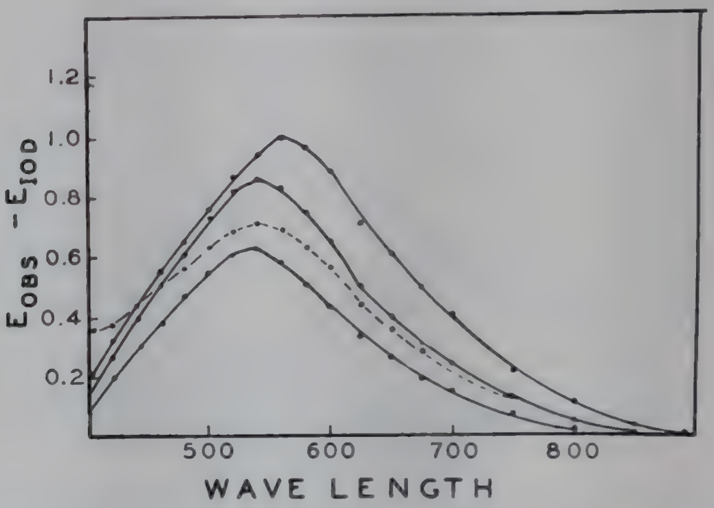

FIG. 14. Absorption spectra of mixtures of amylopectin limit dextrin with synthetic polysaccharides. Average chain length of the synthetic materials from the top downward, 29, 20, 18 glucose units. The dotted line, amylopectin.

SUMMARY

The iodine complexes of amylose, amylopectin, and glycogen have been studied with the aid of a spectrophotometer. From changes in the spectra, which are the same but occur in reverse order during acid hydrolysis and enzymatic synthesis of amylose, it is concluded that the hue formed is mainly dependent on the length of the chain. Chains from 4 to 6 glucose units long give no iodine colors, those from 8 to 12 units give a red color with a peak at 520 mμ, followed by transitional colors until a length of 30 to 35 units is reached, when the iodine color becomes blue with a peak at 600 mμ. Chains as long as 50 to 150 units have been formed by the synthesizing action of potato phosphorylase, and these give absorption spectra closely resembling those of amylose. This relationship between chain length and iodine color has been applied to the branched polysac-

charides. It is estimated that amylopectin has some inner branches as long as 10 units, as shown by the absorption curve of its limit dextrin, while glycogen contains some inner branches as long as 8 units. From the absorption curve of a mixture of amylopectin limit dextrin with synthetic polysaccharides of known chain length, the outer branches of amylopectin are estimated to be about 18 units long.

Hydrolysis of amylose by α-amylase gives changes in the absorption curves from blue to red similar to those produced by acid, although there is some indication that the enzymatic hydrolysis is not completely random. During hydrolysis of amylose by β-amylase the height of the curve steadily decreases, but there is no shift in the peak from 650 mμ. The interpretation seems to be that β-amylase degrades 1 whole molecule completely before attacking another, so that few if any intermediate red staining dextrins are present in the reaction mixture at any one time. The same behavior has been observed when amylose is degraded by potato phosphorylase.

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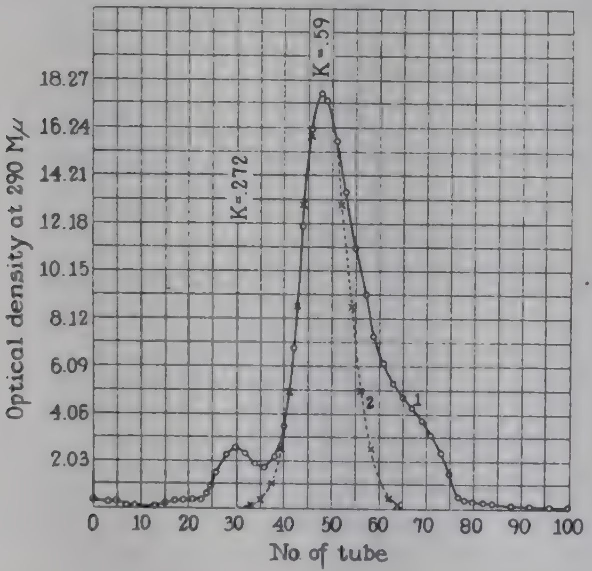
LETTERS TO THE EDITORS

COUNTER-CURRENT DISTRIBUTION OF GRAMICIDIN

Sirs:

As a preliminary in the attempt to extend the method of counter-current distribution to the higher peptides, we have made a preliminary study of several preparations of crystalline gramicidin.[1,2] The figure is a representative result. (1 gm. of substance; 100 transfers with alternate withdrawal[3] in a 54 tube machine; system made from 7, 23, 15, and 15 volumes respectively of water, methanol, chloroform, and benzene.)



A theoretical curve[4] (Curve 2) was fitted to one side of the major component. The result would appear to indicate that at least part of the major band had been separated as a pure component. Redistribution of the material in Tubes 46 and 47 gave essentially a theoretical curve.

When crystallized from acetone, Fractions 26 to 34 combined gave rods which melted on the hot stage at 258–259°; Fractions 40 to 50 gave thin

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[1] Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **141**, 155 (1941).

[2] Preparation kindly supplied by the Wallerstein Laboratories.

[3] Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and du Vigneaud, V., *J. Biol. Chem.*, **168**, 665 (1947).

[4] Williamson, B., and Craig, L. C., *J. Biol. Chem.*, **168**, 687 (1947).

leaves melting at 227–228°. The first component, which was less soluble in organic solvents, will be called gramicidin B. The lower melting component will be called gramicidin A. None of the fractions occurring in the tubes to the right of the A band crystallized sharply. The distribution pattern indicated that all of these tubes contained mixtures. At least two components other than A and B are indicated.

Analytically the various fractions were similar. All gave absorption spectra characteristic of tryptophan in the region of the ultraviolet, but with quantitative differences. Gramicidin A apparently contains the greatest percentage of tryptophan, with B containing approximately 85 per cent as much. The other fractions contained still less tryptophan.

Hydrolytic experiments and paper strip chromatographic analysis[5] have shown gramicidin A and B to contain glycine, alanine, valine, leucine, and tryptophan, but the fractions to the far right of the pattern apparently contain a small percentage of a band corresponding to tyrosine.

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[5] Consdon, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

INFLUENCE OF α-PARTICLES ON THE IMMUNOLOGICAL PROPERTY OF ANTIGEN FILMS

Sirs:

In two previous notes,[1] it was shown that enzymes could act on films of antigen at a distance in spite of intervening blankets of inert material. In the course of the work, which is to appear shortly, some very interesting facts concerning the action of α-particles on antigenic films were observed which have a bearing on long range forces. It was observed that one monolayer of bovine albumin subjected to bombardment by α-particles from a polonium source (5 millicuries per sq. cm.) would completely lose in less than 20 minutes its property of combining with homologous antibody. It took 45 minutes, however, to inactivate one double layer, and 135 minutes were not sufficient to inactivate three double layers or six monolayers. Since the relative loss of energy of α-particles in going through a few layers of proteins (8 A per layer) is negligible, it must be concluded that the amount of energy required to inactivate multilayers is much larger than that required to inactivate a single layer. The loss of the ability of the films to react with homologous antibody following the bombardment with α-particles may be interpreted as due to a rearrangement of groups in the protein molecules. If the layer of protein is directly exposed to the α-particles, rearrangement can occur much more readily than if it is covered by other layers which hinder rearrangement. The degree of freedom of underlying layers is more restricted than that of the top layer, hence the greater stability of multilayers. It was found that thin blankets of Formvar, or heterologous protein, acted as good protectors against inactivation by α-particles, protein films being definitely better protectors than Formvar films. One monolayer of bovine albumin coated with one monolayer of egg albumin kept its immunological reactivity intact following a 20 minute irradiation. The thickness of the layer of antibody specifically adsorbed on six monolayers of bovine albumin was about 40 A after a 2 hour irradiation compared to 180 A without irradiation. But, if a blanket of Formvar 100 A thick was deposited on the layers before α-particle bombardment, the thickness of the antibody layer adsorbed after removing the blanket was not 40 A but 80 A. If six deposited up layers (↑) of bovine albumin covered with a blanket of Formvar 80 A thick are irradiated for 2 hours, they completely lose their capacity for reacting with antibody *through the blanket.* If, however, the blanket is removed before antiserum treatment, 100 A of antibody can still be adsorbed. It was found[2] that without irradiation six up layers of bovine albumin can adsorb 100 A of antibody when they are covered with an 80 A Formvar blanket, and 180 A of antibody if there is no blanket. In other words, following irradiation, a system of six up layers is found to have lost its capacity to react through 80 A of Formvar, although it can still adsorb a large thickness of antibody when the blanket has been removed. It can also be seen that this change cannot be interpreted simply as the inactivation of the one or two topmost layers, leaving the other layers unaltered for the following reasons: (1) A system of two double layers of bovine albumin can adsorb 50 A of antibody when covered with a Formvar blanket 80 A thick, and 100 A when there is no blanket. (2) A system of one double layer or even one monolayer can still adsorb a measurable amount of antibody in spite of a blanket 80 A thick.

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[1] Rothen, A., *J. Biol. Chem.*, **163,** 345 (1946); **167,** 299 (1947).

[2] Rothen, A., *J. Biol. Chem.*, **168,** 75 (1947).

Consequently an irradiated system of six up layers does not behave as if it consisted of a sum of individual active and inactivated layers. Bombardment with α-particles abolishes first the long range reactivity of the system. This might be understood in the sense that the degree of cooperation between the molecules of each layer and between the layers themselves is disrupted by the bombardment. These experiments thus bring new evidence for specific long range forces between macro molecules, and they also open a new vista on the action of α-particles on biological systems.

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GROWTH-PROMOTING ACTIVITY OF GUANINE FOR THE PURINE-DEFICIENT NEUROSPORA 28610*

Sirs:

Analyses of ribonucleic acid for adenine with the adenine-deficient *Neurospora* mutant 28610 have given values about twice as large as those expected from the tetranucleotide theory.[1, 2] Inasmuch as guanine and the pyrimidine components of ribonucleic acid showed no growth-promoting activity for this mutant, the conclusion seemed justified either that the amount of adenine in ribonucleic acid was larger than the commonly accepted value or that another growth-promoting compound, possibly hypoxanthine, was present. We have now found that, although this mutant will not grow on basal medium supplemented with guanine alone, mixtures of adenine and guanine produce more growth than that expected from the amount of adenine used.

Guanine was utilized as efficiently for growth as adenine provided the molar ratio of guanine to adenine did not exceed 0.6. As this ratio was increased beyond 0.6, however, guanine was less efficiently utilized. Over the range from 0.6 to 1.5 equivalents of guanine per equivalent of adenine the effect was relatively constant, giving an amount of growth corresponding to about 1.7 times the true adenine value. When 2 to 3 times as much guanine as adenine was used, the adenine value for growth was approximately twice that expected from the adenine present. The results of a typical experiment in which the amount of adenine used in 25 ml. of basal medium was 0.1 mg. and the molar ratio of guanine to adenine was varied from 0 to 3 are given in the accompanying table.

It is evident from these experiments that this mutant strain cannot be used for adenine assay without qualification if both adenine and guanine are present. As the growth response is additive when the equivalent ratio of guanine to adenine is 0.6 or less, the amount of adenine present can be estimated under these conditions from the amount of growth obtained. An independent method must be used, however, to determine the amount of guanine present, and to show that the amount is less than one-third of the total adenine value indicated by the mold.

The supplementary growth effect of guanine for mutant 28610 accounts in part for the high adenine values found previously for samples of ribonucleic acid by mold assay.[1,2] As the ratio of guanine to adenine in these samples is not within the optimum range for more precise adenine determinations, the amount of adenine in nucleic acid cannot be readily determined by this method.

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* Aided by a grant from the Rockefeller Foundation.

[1] Mitchell, H. K., and Houlahan, M. B., *Federation Proc.*, **3**, 370 (1946).

[2] Loring, H. S., Ordway, G. L., Roll, P. M., and Pierce, J. G., *Federation Proc.*, **6**, 510 (1947).

The fact that the *Neurospora* mutant 28610 utilizes guanine for growth only when adenine is also present indicates that this mold is deficient in the ability to synthesize guanine as well as adenine. When adenine alone is available for growth, it appears likely that it is in part converted to guanine. As shown by Brown *et al.*[3] with adenine containing isotopic nitrogen, the white rat converts adenine in the diet to nucleic acid guanine. The utilization of adenine for guanine synthesis by *Neurospora* as well indicates a more wide-spread significance for this conversion in purine biosynthesis.

Growth-Promoting Activity of Guanine in Presence of 0.1 Mg. of Adenine

Guanine added	Growth of mold	Growth as adenine	Growth (as adenine) / Adenine present
equivalents	*mg.*	*mg.*	
0	11.1	0.098	0.98
0.25	13.8	0.127	1.27
0.50	16.2	0.153	1.53
0.75	17.5	0.168	1.68
1.00	17.0	0.161	1.61
1.25	17.8	0.170	1.70
1.50	17.3	0.165	1.65
2.00	20.5	0.205	2.05
3.00	19.1	0.196	1.96

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[3] Brown, G. B., Roll, P. M., and Plentl, A. A., *Federation Proc.*, **6**, 517 (1947).

THE REVERSAL OF THE BACTERIOSTATIC ACTION OF STREPTOMYCIN BY UREA

Sirs:

The bacteriostatic effect of streptomycin on *E. coli* and *M. tuberculosis* 607 and H37RV can be antagonized by a number of purines and pyrimidines. These include xanthine, uric acid, allantoin, alloxan, and parabanic acid. It seemed possible that these compounds might give rise to urea. If such were the case, urea should antagonize the effect of streptomycin and the effect should be demonstrable sooner than when the urea precursors are used. These two conditions were fulfilled and the table shows the reversal of the streptomycin inhibition by urea on the growth of *M. tuberculosis* 607. Streptomycin HCl (Merck) was used and the organisms grown in 10 cc. of Tween medium[1] without albumin. Tubes were inoculated with 0.5 cc. of a 1:20 dilution of a 6 day-old culture grown in the same medium. Turbidities were read in the Evelyn photoelectric colorimeter with a No. 420 filter.

Reversal of Bacteriostatic Action of Streptomycin by Urea

	Density* at 48 hrs. (*M. tuberculosis* 607 in Tween medium)						
Streptomycin concentration, micrograms per cent	Urea concentration; mg. per cent						
	0	3.1	6.2	12.5	25	50	100
100	0	0	0	2.4	7.9	8.5	9.06
50	0	0	2.4	8.0	9.1	9.6	9.6
10	0.5						
5	6.4						
1	9.1						
0	9.6			8.5	9.3	9.1	9.6

* Density = $(2 - \log T) \times 100$.

If asparagine in the Tween medium is replaced by 0.1 per cent urea as the nitrogen source, 700 micrograms per cent of streptomycin are required to inhibit growth completely, whereas 25 micrograms per cent produce this effect in the asparagine medium. During growth urea disappears and, since these bacteria apparently contain no urease, urea may be used as such for synthesis of purine and pyrimidines needed by the organism. Thiourea will not reverse the streptomycin inhibition and is itself bacteriostatic in concentrations of 50 to 100 mg. per cent.

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[1] Dubos, R. J., *et al.*, *Am. Rev. Tuberc.*, **54**, 204 (1946).

Similar results were obtained with *E. coli* (American Type Culture Collection, No. 6522) grown on a synthetic medium.[2] Preliminary results with *M. tuberculosis* H37RV indicate that the same mechanism may occur in these organisms as well.

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[2] Kohn, H. I., and Harris, J. S., *J. Pharmacol. and Exp. Therap.*, **73**, 343 (1941).

INTRACELLULAR STRUCTURES AND THE FATTY ACID OXIDASE SYSTEM OF RAT LIVER

Sirs:

In an earlier investigation it was shown that enzymatic oxidation of octanoate occurred in saline suspensions of washed particulate matter separated from saline homogenates of rat liver by centrifugation, when these were supplemented with adenosine triphosphate and Mg^{++}.[1] These preparations also catalyze the reactions of the Krebs tricarboxylic acid cycle. Further study of the properties of the enzyme complex has revealed that the oxidation of octanoate requires the presence of cytochrome *c*, inorganic phosphate, certain neutral salts such as NaCl, KCl (or certain nonelectrolytes such as sucrose), and catalytic amounts of malate or oxalacetate, in addition to adenine nucleotide and Mg^{++}.[2,3]

Fatty Acid Oxidase Activity of Subcellular Fractions

Warburg vessels contained final concentrations of 0.0005 M ATP, 0.005 M $MgSO_4$, 0.05 M KCl, 1×10^{-5} M cytochrome *c*, 0.01 M phosphate buffer, pH 7.4, 0.0005 M *l*-malate, and 0.001 M octanoate or water, 0.90 ml. of water suspensions of fractions indicated, and water to make 5.0 ml. $T = 30°$; time, 1.0 hour. Data in micromoles.

Enzyme source	Octanoate	O_2 uptake	Octanoate removed	Acetoacetate formed
Saline-washed particles	–	3.1		0.1
	+	13.6	4.9	7.9
Mitochondria	–	3.3		0.1
	+	15.4	5.0	6.9
Nuclei	+	1.5		0.1
Microsomes	+	2.8		0.1
Mitochondria from saline-washed particles	–	1.7		0.2
	+	14.9	5.0	6.1

The behavior of the enzyme preparations toward water and solutions of salts or non-electrolytes suggested that the activity was dependent on the integrity of some organized intracellular structure present in the preparations. Accordingly, we have examined the fatty acid oxidase activity of various particulate fractions such as nuclei, "large granules," microsomes, etc., which are separable from rat liver homogenates by differential centrifugation. We have found that all of the demonstrable fatty acid oxidase activity of rat liver can be recovered in that fraction of 30 per cent sucrose homogenates of rat liver stated by Hogeboom *et al.*[4] to consist of morphologically intact mitochondria, free of extraneous intracellular elements. No activity could be detected in other fractions. Typical data are shown in the table. These preparations also catalyze reactions of the Krebs cycle with coupled phosphorylations.

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[1] Lehninger, A. L., *J. Biol. Chem.*, **161**, 437 (1945).

[2] Lehninger, A. L., and Kennedy, E. P., unpublished work.

[3] Potter, V. R., *J. Biol. Chem.*, **163**, 437 (1946).

Since these findings suggested that the saline-washed material previously studied[1] contained mitochondria, such a preparation was subjected to the same procedure used for the isolation of mitochondria from whole liver. The material recovered appeared to be identical with mitochondria obtained from whole liver and was highly active, as the data in the table show.

The localization of such highly integrated enzyme systems in organized intracellular elements is not only of cytochemical interest but poses certain problems in the further study of the mechanism of these oxidations.

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[4] Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *Proc. Soc. Exp. Biol and Med.*, **65**, 320 (1947).

HE ANTAGONISM OF ANTIBACTERIAL ACTION OF ATABRINE

rs:

Naturally occurring polyamines and several B vitamins can partially tagonize the inhibitory effects of atabrine in the growth of *Escherichia li.*[1, 2] It was indicated that natural materials contain a substance or bstances capable of completely negating the inhibitory effects of atabrine.[1]

Addition	Atabrine concentration per tube		
	0	0.5 mg.	0.75 mg.
	Time required for visible growth		
	hrs.	*hrs.*	*hrs.*
one	4.5	44	>48
mg. Bacto-peptone*	4.5	20	>48
" enzymic lactalbumin†	4.5	4.5	6.0
" " " †	4.5	4.5	4.5
2 " " " ash equivalent to 5 mg. original material	4.5	4.5	6.5
4 mg. enzymic lactalbumin; ash equivalent to 10 mg. original material	4.5	4.5	5.5
2 mg. $CaCl_2$ ($\approx$ 72.2 γ Ca)	4.5	5.0	6.5
4 " " ($\approx$ 144.4 " ")	4.5	4.5	4.5
2 " $MgCl_2$ ($\approx$ 51.1 " Mg)	4.5	5.0	7.5
4 " " ($\approx$ 102.2 " ")	4.5	4.5	6.0
4 " NaCl	4.5	44	>48
4 " KH_2PO_4	4.5	40	>48

Basal medium, Bacto-peptone 1 per cent, NaCl 0.6 per cent, glucose 0.2 per cent; olume 5.0 ml.; initial pH after additions 7.45; incubation temperature, 30°; inoculum, 1 drop of 24 hour broth culture of *E. coli* diluted 1:1000; ash content of enzymic ctalbumin 4 per cent; all salts Mallinckrodt, reagent grade.

* Contains 0.2 γ per mg. of Ca; 0.6 γ per mg. of Mg.

† Contains 12.1 γ per mg. of Ca; 2.0 γ per mg. of Mg.

This study has recently been resumed, and in a survey of natural materials for the occurrence of atabrine antagonists, high activity was found a sample of enzymatically digested lactalbumin.[3] The activity of this aterial was not destroyed by prolonged autoclaving at pH 3.0 or 9.0. t the suggestion of Dr. John C. Keresztesy of this laboratory, a portion f it was ashed. The greater part, if not all, of the activity has been found n the ash. The results are shown in the table.

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[1] Silverman, M., and Evans, E. A., Jr., *J. Biol. Chem.*, **154**, 521 (1944).

[2] Miller, A. K., and Peters, L., *Arch. Biochem.*, **6**, 281 (1945).

[3] Lactalbumin hydrolysate (enzymic), Nutritional Biochemicals Corporation.

Inasmuch as calcium as $CaCl_2$ effectively antagonizes the growth inhibition of atabrine, it appears that the activity of the enzymic lactalbumin and its ash, under the indicated experimental conditions, is largely due to its high calcium content. Magnesium as the chloride is about as effective as calcium.

Under the growth conditions described, calcium will also antagonize the growth inhibition of *E. coli* by the cyanine dye, 1,1′-dimethyl-2,2′-cyanine chloride.[4]

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[4] Parke, Davis and Company; Eastman Kodak Company.

AN IN VIVO EFFECT OF PTEROYLGLUTAMIC ACID UPON TYROSINE METABOLISM IN THE SCORBUTIC GUINEA PIG

Sirs:

Phenolic compounds are increased in the urine of patients with untreated pernicious anemia[1] and liver suspensions from pteroylglutamic acid-deficient rats are better able to oxidize tyrosine after the addition of PGA.[2] These observations led us to study the effect of this vitamin upon the metabolism of tyrosine in the scorbutic guinea pig.[3]

Agent	Daily dose	Urinary excretion*	Successive days								
			Pretreatment		Treatment				Post treatment		
			−2	−1	1	2	3	4	5	6	7
	mg.										
Ascorbic acid†	10	"Tyrosyl"	59.8	46.1	15.3	7.5	3.4	4.4	3.9	21.1	43.9
		Keto acid	34.5	22.0	6.0	0.6	0.2	0.2	0.1	7.2	25.2
PGA	15	"Tyrosyl"	67.7	65.4	58.9	10.7	5.8	7.9	5.6	5.1	51.7
		Keto acid	30.0	24.3	31.4	3.3	0.5	0.6	0.3	0.8	28.2
"	15	"Tyrosyl"	65.5	63.5	64.6	23.9	5.6	5.1	6.2	12.6	44.7
		Keto acid	36.8	25.9	35.7	19.0	0.2	0.1	0.1	4.7	23.9
"	5	"Tyrosyl"	43.5	31.8	57.8	32.2	4.1	10.3	32.6	26.3	32.0
		Keto acid	20.8	11.6	28.0	14.6	0.1	2.4	14.9	10.7	15.0
"	5	"Tyrosyl"	75.5	94.5	83.0	41.2	61.5	60.3	52.4	49.1	64.6
		Keto acid	47.3	42.8	48.9	23.0	26.0	27.2	25.3	22.1	33.0

* "Tyrosyl" was measured as tyrosine, and keto acids were measured as phenylpyruvic acid. Both are expressed as per cent of added tyrosine ingested.

† Average of data on three guinea pigs.

Albino guinea pigs of both sexes, 150 to 350 gm. in weight, were fed a scorbutigenic diet[4] to which had been added 5 per cent L-tyrosine. The 24 hour urinary excretion of tyrosyl derivatives[5] and keto acids[6] was determined. After stabilization of the excretion of these metabolites at abnormally high levels, PGA or ascorbic acid was given orally for 4 consecutive days. During the experimental period the food intakes of the animals in the several groups were comparable. Results of one set of experiments are tabulated in the table. The decrease in excretion has been observed in ten successive curative attempts. The poorest result observed is included in the table. In two additional groups of guinea pigs on the same diet 5 mg. of PGA subcutaneously or 10 mg. of ascorbic acid orally were administered 3 times a week in order to test whether the defect could be prevented. From the 5th to the 26th day on the diet, which afforded an average daily intake of about 450 mg. of tyrosine, 24 hour collections of urine were made at intervals of 4 to 7 days. In the control group the excretion of "tyrosyl" and keto acid was 45 and 21 per cent of added tyrosine respectively; the corresponding values in the group receiving ascorbic acid were 8.2 and 1.6 per cent, and in the group receiving PGA they were 19.8 and 5.4 per cent.

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[1] Swendseid, M. E., Burton, I. F., and Bethell, F. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 202 (1943).

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[3] Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135**, 251 (1940).

[4] Darby, W. J., DeMeio, R. H., Bernheim, L. C., and Bernheim, F., *J. Biol. Chem.*, **158**, 67 (1945).

[5] Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).

[6] Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).

This defect in tyrosine metabolism is influenced by either PGA or ascorbic acid.

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URIC ACID DIABETES

Sirs:

Diabetogenic doses of alloxan lower blood reduced glutathione levels in rats from an average of 43 to 25 mg. per 100 gm. of blood.[1] Ninhydrin, though non-diabetogenic,[2] also lowers blood glutathione to very low levels.[1] These observations suggested that uric acid, parenterally administered, might exert a diabetogenic action if blood reduced glutathione were lowered before injection. This has been tested by feeding adult rabbits the methionine- and cystine-deficient diet described by Haag and Wright,[3] modified by replacement of yeast by synthetic vitamins and by inclusion of 0.1 per cent ascorbic acid.

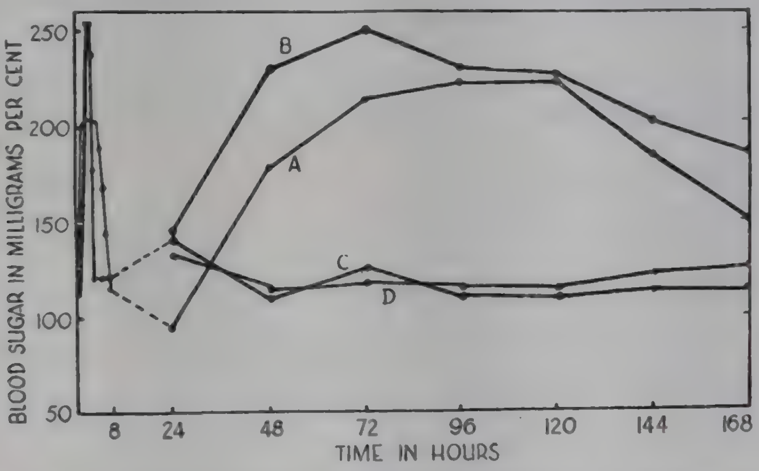

FIG. 1. Curve A, average response of four methionine- and cystine-deficient rabbits to an intraperitoneal injection of 1 gm. per kilo of uric acid. Curve B, average response of three normal rabbits to an intravenous injection of 200 mg. per kilo of alloxan monohydrate. Curve C, average response of four rabbits on the deficient diet, but given a methionine supplement, to an intraperitoneal injection of 1 gm. per kilo of uric acid. Curve D, blood sugar levels of a methionine- and cystine-deficient rabbit fed a standard laboratory diet and fresh lucerne.

During 6 to 7 weeks the blood glutathione fell from an average initial value of 38 to 18 to 23 mg. per cent. In one case the lowering was hastened by eliminating protein from the diet for 2 weeks. Intraperitoneal injection of 1 gm. per kilo of uric acid into these deficient animals caused an initial hyperglycemia, lasting about 8 hours. After 24 hours the blood sugar levels were at or below the control level. From the 24th to the 48th hour blood sugar slowly rose to hyperglycemic levels (Fig. 1, Curve A). A standard laboratory diet and fresh alfalfa were then fed to the animals. They remained hyperglycemic and glycosuric for the next 4 to 5 days. Thereafter normal blood sugar levels were regained.

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[1] Brückmann, G., and Wertheimer, E., *J. Biol. Chem.*, **168**, 241 (1947).

[2] Hidy, P. H., *J. Biol. Chem.*, **163**, 307 (1946).

[3] Haag, J. R., and Wright, L. D., *J. Nutr.*, **19**, 563 (1940).

The same sequence of events followed intravenous injection of 200 mg. per kilo of alloxan monohydrate into normal rabbits on the standard diet (Curve B).

The blood reduced glutathione of rabbits fed the deficient diet plus a supplement of 0.2 per cent methionine remained above 30 mg. per cent. Injection of uric acid into these animals induced an initial hyperglycemia lasting about 4 hours but no prolonged secondary hyperglycemia developed (Curve C). Feeding of the standard diet and alfalfa to a deficient rabbit had no effect on blood sugar levels.

It is with pleasure that the writer acknowledges his indebtedness to Dr. F. W. Clements for his advice on diets.

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THE INHIBITORY ACTION OF IODIDE UPON ORGANIC BINDING OF IODINE BY THE NORMAL THYROID GLAND

Sirs:

The dramatic but paradoxical action of iodine in inhibiting thyroid activity in Graves' disease has not as yet received a satisfactory explanation. It has generally been assumed that this inhibitory effect occurs only in the hyperactive gland. It is shown here, however, that *plasma iodine* in large enough concentrations will block organic binding of iodide in the *normal* thyroid gland. Thus thyroid tissue is inhibited by iodine regardless of its state of activity.

Each thyroid value represents the average of five or six determinations on as many rats.

Amount of I^{127} injected	Interval after injection									
	2.5 hrs.		5 hrs.		12 hrs.		25 hrs.		50 hrs.	
	Total plasma I^{127}	Injected iodide organically bound by thyroid	Total plasma I^{127}	Injected iodide organically bound by thyroid	Total plasma I^{127}	Injected iodide organically bound by thyroid	Total plasma I^{127}	Injected iodide organically bound by thyroid	Total plasma I^{127}	Injected iodide organically bound by thyroid
γ	γ *per cent*	γ I^{127}	γ *per cent*	γ I^{127}	γ *per cent*	γ I^{127}	γ *per cent*	γ I^{127}	γ *per cent*	γ I^{127}
10	4.9	2.1	4.0	5.7	4.2	4.4	3.8	3.1	4.6	2.0
50	41	0.19	34	0.19	15	3.2	7.1	3.8	5.1	3.5
100	59	0.12	46	0.24	22	1.9	18	3.4	11	4.9
200	108	0.12	93	0.12	23	0.24	6.7	6.1	6.0	6.8

Rats were injected intraperitoneally with either 10, 50, 100, or 200 γ of iodine as KI labeled with I^{131}. At the intervals indicated in the table, blood was removed by heart puncture from the five or six rats of each group and their plasma pooled for duplicate analyses of total iodine.[1] Thyroid glands were excised, weighed rapidly, and ground with 1 ml. of cold 10 per cent trichloroacetic acid in an all-glass homogenizer. The precipitate was then washed twice with 2.5 ml. of 5 per cent trichloroacetic acid and dissolved in a minimal amount of 2 N NaOH. Radioactivity of the organic fraction (trichloroacetic acid-insoluble) was measured with the aid of a Geiger-Müller counter. The amount of I^{127} organically bound was obtained by multiplying the numerical proportion of the radioactivity recovered in the organic fraction by the number of micrograms of I^{127} injected.

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[1] Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **163**, 313 (1946).

As early as 2.5 hours, 2 γ of iodide were organically bound by the thyroid glands of the rats that had been injected with the smallest amount of iodide, namely 10 γ. In contrast, the rats that had received 50, 100, or 200 γ failed to bind organically significant amounts of the administered iodide even as late as 5 hours; *i.e.*, at a time when the 10 γ group had already bound approximately 6 γ of the administered iodide. The most striking effect was seen in the 200 γ group in which practically none of the injected iodide had been converted to organic iodine as late as 12 hours after the injection. Thus, during the early intervals, the thyroids of rats that had received 10 γ of iodide incorporated far greater amounts into the organic fraction than did those that had available 5 to 20 times as much iodide.

The inhibitory action observed here is related to the level of plasma iodine, for, when the latter reached values in the neighborhood of 15 to 25 γ per cent, organically bound iodine rapidly appeared in the thyroids (see the table).

These findings as well as the *in vitro* effects reported earlier[2] point directly to an interference by excess iodide upon the mechanism responsible for organic binding of iodide. There appears to be escape from this inhibition when the total plasma iodine drops to values near 15 to 25 γ per cent.

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[2] Morton, M. E., Chaikoff, I. L., and Rosenfeld, S., *J. Biol. Chem.*, **154**, 381 (1944.)

AN α-CELL HORMONE OF THE ISLETS OF LANGERHANS*

Sirs:

A second hormone of the islets of Langerhans, elaborated by the α-cells and acting antagonistically to insulin, has long been suggested by (*a*) the many reports in the early insulin literature of a preliminary hyperglycemic action of certain relatively crude preparations of insulin protein,[1] (*b*) the more recently recognized histological appearance of the α-cells, which exhibit the granulation characteristic of endocrine function, (*c*) the reduction of the hyperglycemia, the development of ketosis, and the fatal termination which follow removal of the pancreas in the alloxan-diabetic dog,[2] and (*d*) clinical data in "diabetes mellitus" which cannot be reconciled with absolute hypoinsulinism.

In a search for active extracts of the suspected α-cell hormone, we have examined the physiological properties of various pancreatic fractions in the course of separation of crystalline zinc insulin[3] and have succeeded consistently in obtaining preparations which cause liver glycogenolysis (*in vivo* and *in vitro*) and hyperglycemia.

The supernatant solution from the first isoelectric precipitation of insulin[3] was concentrated 10-fold *in vacuo* at room temperature and the insulin inactivated by boiling (20 minutes) at pH 9.5 to 9.7 (with rejection of the formed precipitate. The table shows the effect of intraperitoneal injection of the filtrate into intact fed rats (2 ml. (14 mg. of nitrogen) *per* animal; each figure represents the average of seven animals (glycogen) or four animals (sugar); the controls were injected with 2 ml. of a 1 per cent solution of casein).

The observed maximum differences are statistically significant and the above pattern has been reproduced many times with different strains of rats and with different pancreas extracts. In efforts to purify the active

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We are greatly indebted to Dr. D. A. Scott and Dr. A. Fisher, Connaught Laboratories, Toronto, for the generous provision of starting materials, and to Mr. D. Macfarlane for skilful assistance; in support of the investigation we gratefully acknowledge the help of the Banting Research Foundation, the Associate Committee on Medical Research of the National Research Council, Ottawa, the Committee for Research in Endocrinology of the National Research Council, Washington, and the Sugar Research Foundation.

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[2] Thorogood, E., and Zimmermann, B., *Endocrinology*, **37**, 191 (1945).

[3] Romans, R. G., Scott, D. A., and Fisher, A. M., *Ind. and Eng. Chem.*, **32**, 908 (1940).

material, comparable potency has been attained in preparations containing 2 mg. of nitrogen *per* rat dose. Stored without preservative at 4° and pH 9.5, crude extracts lose considerable activity in 8 to 10 days; purified preparations are more labile (1 to 5 days).

Time after injection	Liver glycogen		Blood sugar	
	Control	Experimental	Control	Experimental
min.	*gm. per cent*	*gm. per cent*	*mg. per cent*	*mg. per cent*
0	3.78	3.78	106	106
20	3.75	2.55	112	162
40	3.13	1.75	105	193
60	3.08	1.22	100	160
90	2.65	1.54	105	122

Incubation (37°; 1 hour) of active extract with rat liver slices (Ringer-phosphate medium) increases oxygen consumption and releases 20 to 30 per cent more glucose than untreated controls. Large doses fail to alter the blood pressure of the atropinized cat. Similarly prepared extracts of muscle or liver could not be shown to affect liver glycogen or blood sugar. These control observations tend to preclude non-specific action, or mediation through the adrenal or anterior pituitary.

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